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(71) Applicant: TANOX, INC. [US/US]; 10301 Stella Link, Houston, TX 77025 (US).

(72) Inventors: THOMAS, David; 1920 Albans Rd., Houston, TX 77005 (US). DE BOER, Mark; Naarderweg 20, NL-1261 BT Blaricum (NL). RES, Pieter, C., J., M.; Bloemveldlaan 49, NL-2015 HC Haarlem (NL). SIMONS, Peter, J.; Leidsestraat 43, NL-2182 DH Hillegom (NL).

(74) Agent: MIRABEL, Eric, P.; Tanox, Inc., 10301 Stella Link, Houston, TX 77025 (US).

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(54) Title: CD40-BINDING APC-ACTIVATING MOLECULES

(57) Abstract: Disclosed are agonist anti-CD40 molecules, including monoclonal antibodies, which can bind to and stimulate professional and non-professional human antigen-presenting cells ("APCs"), enhance the stimulatory effect of CD40L on CD40 positive cells and/or induce phenotypical maturation of monocyte derived dendritic cells. Several such monoclonal antibodies are provided, and cell lines producing them have been deposited at the American Type Culture Collection.

CD40-BINDING APC-ACTIVATING MOLECULES

Field of the Invention

This invention relates to a series of novel molecules and monoclonal antibodies that bind to and stimulate antigen presenting cells via the CD40 receptor expressed on such antigen presenting cells.

Background of the invention

Activation of the immune system:

The immune system is capable of killing autologous cells when they become infected by virus or when they transform into cancer cells. Such a potentially dangerous mechanism is under tight control. When they have not yet encountered their specific antigen, the immune system's T-killer cells (CTL) circulate as inactive precursors. To be activated, the precursor T-killer cell must recognize its specific antigen peptide, presented by MHC class I molecules on professional antigen presenting cells (APC). This antigen specific cellular interaction is, however, not enough to fully activate the CTL, notwithstanding the co-stimulatory signals from the APC.

Until recently it was believed that a T-helper cell that recognises the same antigen on the same APC as the CTL is needed to fully activate the CTL. Upon activation, the specific T-helper cell would supply cytokines such as IL-2 needed for the activation of the CTL. Guerder and Matzinger (J. Exp. Med. 176:553 (1992)), however, proposed the "licensing" model for CTL activation. In this model it was suggested that the T-helper cell, when recognising its antigen on a professional APC, would deliver an activation signal to the APC that as

a result would be able to subsequently activate a CTL without the need for the T-helper cell to be present. Recently, the molecular mechanism of the licensing model was elucidated. Schoenberger et al. (Nature 393:480 (1998)), described the crucial role of the CD40L-CD40 pathway in the licensing model. Activation of the T-helper cell by the dendritic cell (DC) results in the up-regulation of the CD40L, which subsequently provides the signal that empowers the DC for CTL priming by triggering the CD40 molecule on the DC.

DC circulate through and are resident in the body tissues and at sites of antigen deposition or introduction. After taking up antigens, they migrate to the draining lymph nodes where they present antigen to the T cells. It is well known that a DC needs to be activated to perform optimally. Resting DC express only low levels of MHC and co-stimulatory molecules and are poor stimulators of T cells. DC can be activated by inflammatory cytokines and bacterial products, which results in up-regulation of MHC and co-stimulatory molecules. Therefore, DC that have encountered antigens under inflammatory conditions will readily activate T-helper cells when they arrive in the draining lymph nodes. It is thus very likely that the combination of inflammatory cytokines at the site of antigen uptake and the CD40L-CD40 interaction during the T-helper cell interaction result in an optimal capacity to license the DC for CTL activation.

The CD40 molecule and the TNF receptor family:

The CD40 molecule belongs to the TNF receptor family of type I transmembrane proteins. The members of this gene family (which include among others, the two receptors for TNF, the low-affinity nerve

growth factor receptor and the T cell activation antigen CD27, CD30, and CD95) are characterized by sequence homology in their cysteine-rich extracellular domains (Armitage et al., Current Opinion in Immunology 6:407 (1994)). The known ligands for the members of the TNF receptor family are homologous as well. Although TNF-α is a soluble cytokine, it is initially synthesized as a membrane associated molecule. Most of the members of the TNF/CD40L receptor and the TNF/CD40 families are type II trans-membrane proteins. These include: hTNF-α, hLT, hLT-β, hCD40L, hCD27L, hCD30L, cfECP1, myx VRh, mCD30, hCD27, hFas, m4-1BB, rOX-40, hTNFR-h, hTNFR-II, hTNFR-1 and hLNGFR. CD40 is best known for its function in B-cell activation. The molecule is constitutively expressed on all B cells. CD40L-CD40 interaction can stimulate the proliferation of purified B cells and, in combination with cytokines, mediate immunoglobulin production. Recent studies indicate that the distribution of the CD40 molecule is not as restricted as was originally postulated. Freshly isolated human monocytes express low levels of the CD40 molecule, which can be up-regulated by culturing them in the presence of IFN-α (Alderson et al., J. Exp. Med. 178:669 (1993)). Stimulation of monocytes via CD40 results in the secretion of pro-inflammatory cytokines such as IL-1 and TNF-α, toxic free radical intermediates such as nitric oxide and up-regulation of the B7 co-stimulatory molecules. Human DC isolated from peripheral blood can also express the CD40 molecule (Caux et al., J. Exp. Med. 180:263 (1994)). Ligation of CD40 on DC results in enhanced survival of these cells when cultured *in vitro*. As with monocytes, stimulation of DC via CD40 results in secretion of pro-inflammatory cytokines such as IL-12 and TNF-α and up-regulation of the CD80/86 co-stimulatory molecules. In addition, it was recently demonstrated that activation of

CD40 induces the capacity to stimulate the activation of killer T cells (Schoenberger et al., Nature 393:480 (1998)). Accordingly, activating CD40 by binding it with a ligand, such as an antibody, would induce a number of humoral and cytotoxic effects, useful in inhibiting tumors.

Summary of the invention

The invention includes molecules able to bind to and activate CD40 expressed on both professional and non-professional APCs. These agonistic molecules, following binding to a cell surface receptor, induce intracellular signal transduction, leading to the activation of the APCs expressing CD40. The molecules of the invention include monoclonal antibodies, fragments thereof, peptides, oligonucleotides, and other chemical entities. Also included are peptides and genes inducing expression of anti-CD40 antibodies.

Such molecules can be used in combination, or in a bispecific or multivalent form, including as bispecific antibodies, to cross-link CD40 on the same cell, or to cross-link CD40 present on different cells. Either such cross-linking could cause a synergistic or additive agonistic effect.

Brief Description of the Drawings

Fig. 1 shows the induction of maturation of monocyte derived DC by anti-CD40 monoclonal antibodies. Monocyte derived immature dendritic cells were cultured for two days in the presence of anti-CD40 monoclonal antibodies or isotype matched control antibodies and then studied by FACS analysis for up-regulation of expression of CD80 and down-regulation of the mannose receptor. Shown are the combined results of several experiments with the percentage of cells expressing

CD83 in Fig1a and the relative decrease in mean fluorescence intensity (MFI) of the mannose receptor expression in unstimulated cells (MFI arbitrary taken as a value of 100) compared to stimulated cells in Fig 1b.

Fig. 2 shows induction of maturation of monocyte derived DC by anti-CD40 monoclonal antibodies (clones 7, 15, 21, 48, 64 and 70). Monocyte derived immature dendritic cells were cultured for two days in the presence of anti-CD40 monoclonal antibody or isotype matched control antibodies and then studied by FACS analysis for up-regulation of expression of CD80, CD83 and CD86 and down-regulation of expression of the mannose receptor. Data of one representative experiment are shown: the CD80 (Fig. 2a), CD86 (Fig. 2c) and mannose receptor (Fig. 2d) expression are indicated as mean fluorescence intensity, whereas CD83 is indicated as the percentage of cells expressing this marker for mature dendritic cells (Fig. 2b).

Fig. 3 shows induction of IL-12p70 secretion by monocyte derived DC after stimulation with CD40 agonist antibodies and IFN-γ. Monocyte derived immature DC were cultured for two days in the presence of anti-CD40 monoclonal antibodies or isotype control antibodies alone or in combination with IFN-γ. Induction of IL-12p70 production requires the combination of two different stimuli.

Fig. 4 shows IL-12p70 production induced by CD40 agonist antibodies and IFN-γ is blocked by pre-incubation with CD40-Fc. Pre-incubation of the CD40 agonist antibodies with an excess of CD40-Fc abolished the

ability of the anti-CD40 monoclonal antibodies to induce, in combination with IFN-g, IL-12 production in monocyte derived DC.

Fig. 5 shows CD40 agonist monoclonal antibodies prime DC with an enhanced ability to induce CD8+ T cell responses. Monocyte derived DC were either left un-stimulated, or pre-activated with CD40 agonist antibody with or without IFN-γ and subsequently co-cultured with purified autologous CD8+ T cells in the presence of a flu matrix peptide representing a dominant HLA-A2 restricted epitope recognized by CD8+ T cells. The induction of CD8+ T cell responses by CD40 activated DC was studied by analyzing both the expansion of flu peptide specific CD8+ T cells 9 (Fig. 5a) and the increase in CD8+ T cells that produce IFN-γ (Fig. 5b).

Detailed description of the invention

The molecules described and claimed include monoclonal antibodies, fragments thereof, peptides and other chemical entities. Monoclonal antibodies can be made by the conventional method of immunization of a mammal, followed by isolation of the B cell producing the monoclonal antibodies of interest and fusion with a myeloma cell. The preferred monoclonal antibodies include chimeric antibodies, humanized antibodies, human antibodies, DeImmunized™ antibodies, single-chain antibodies and fragments, including Fab, $F(ab')_2$, Fv and other fragments which retain the antigen binding function of the parent antibody. Single chain antibodies ("ScFv") and the method of their construction are described in U.S. Patent No. 4,946,778.

Chimeric antibodies are produced by recombinant processes well known in the art, and have an animal variable region and a human constant region. Humanized antibodies correspond more closely to the sequence of human antibodies than do chimeric antibodies. In a humanized antibody, only the complementarity determining regions (CDRs), which are responsible for antigen binding and specificity, are non-human derived and have an amino acid sequence corresponding to the non-human antibody, and substantially all of the remaining portions of the molecule (except, in some cases, small portions of the framework regions within the variable region) are human derived and have an amino acid sequence corresponding to a human antibody. *See* L. Riechmann et al., Nature; 332: 323-327 1988; U.S. Patent No. 5,225,539 (Medical Research Council); U.S. Patent Nos. 5,585,089; 5,693,761; 5,693,762 (Protein Design Labs, Inc.).

Human antibodies can be made by several different methods, including by use of human immunoglobulin expression libraries (Stratagene Corp., La Jolla, California; Cambridge Antibody Technology Ltd., London, England) to produce fragments of human antibodies (V_H, V_L, Fv, Fd, Fab, or $(Fab')_2$), and use of these fragments to construct whole human antibodies by fusion of the appropriate portion thereto, using techniques similar to those for producing

chimeric antibodies. Human antibodies can also be produced in transgenic mice with a human immunoglobulin genome. Such mice are available from Abgenix, Inc., Fremont, California, and Medarex, Inc., Annandale, New Jersey. In addition to connecting the heavy and light chain Fv regions to form a single chain peptide, Fab can be constructed and expressed by similar means (M.J. Evans et al., J. Immunol. Meth., 184: 123-138 1995).

DeImmunized™ antibodies are antibodies in which the potential T cell epitopes have been eliminated, as described in International Patent Application PCT/GB98/01473. Therefore, immunogenicity in humans is expected to be eliminated or substantially reduced when they are applied *in vivo*.

All of the wholly and partially human antibodies described above are less immunogenic than wholly murine or non-human-derived antibodies, as are the fragments and single chain antibodies. All these molecules (or derivatives thereof) are therefore less likely to evoke an immune or allergic response. Consequently, they are better suited for *in vivo* administration in humans than wholly non-human antibodies, especially when repeated or long-term administration is necessary, as may be needed for treatment of psoriasis or other inflammatory skin conditions.

Bispecific antibodies can be used as cross-linking agents between CD40 of the same cell, or CD40 on two different cells. Such bispecific antibodies would have one specificity for each of two different epitopes on CD40. Bispecifics in which one specificity is a strong activator of binding of sCD40L to CD40, and one specificity is a partial or non-inhibitor of binding of sCD40L to CD40, could synergize the agonistic effect on cross-linking.

These antibodies and the method of making them are described in U.S. Patent No. 5,534,254 (Creative Biomolecules, Inc.). Different embodiments of bispecific antibodies described in the patent include linking single chain Fv with peptide couplers, including Ser-Cys, $(Gly)_4$-Cys, $(His)_6$-$(Gly)_4$-Cys, chelating agents, and chemical or disulfide couplings including bismaleimidohexane and bismaleimidocaproyl.

Non-antibody molecules can be isolated or screened from compound libraries by conventional means. An automated system for generating and screening a compound library is described in U.S. Patent Nos. 5,901,069 and 5,463,564. A more focused approach involves three-dimensional modeling of the binding site, and then making a family of molecules which fit the model. These are then screened for those with optimal binding characteristics.

Another approach is to generate recombinant peptide libraries, and then screen them for those which bind to the epitope of CD40 of interest. See, *e.g.*, U.S. Patent No. 5,723,322. This epitope is the

same as that bound by the monoclonal antibodies described in the examples below. Molecules can, in fact, be generated or isolated with relative ease in accordance with techniques well known in the art once the epitope is known.

Another approach is to induce endogenous production of the desired anti-CD40 antibodies, by administering a peptide or an antibody which induces such production, or through gene therapy, where a gene encoding an appropriate anti-CD40 molecule or a fragment thereof is administered. The method of making and administering any of these molecules is well known in the art.

The molecules can be administered by any of a number of routes. In the case of peptides and antibodies, because they are subject to degradation in the gastro-intestinal tract, they would preferably be injected. Other compounds of the invention could also be injected. The injections could be intra-muscular, intra-venous or sub-cutaneous.

Non-peptide molecules of the invention could be administered orally, including by suspension, tablets and the like. Liquid formulations could be administered by inhalation of lyophilized or aeorosolized microcapsules. Suppositories could also be used.

Additional pharmaceutical vehicles could be used to control the duration of action of the molecules of the invention. They could be entrapped in microcapsules prepared by coacervation techniques or by interfacial polymerization (hydroxymethylcellullose or gelatin microcapsules) in colloidal drug delivery systems (for example, liposomes, albumin microspheres, micro-emulsions, nanoparticles and nanocapsules) or in macro-emulsions.

Excipients, for example, salts, various bulking agents, additional buffering agents, chelating agents, antioxidants, cosolvents and the like can be included in the final formulation. Specific examples include tris-(hydroxymethyl) aminomethane salts ("Tris buffer") and disodium edetate.

The dosage and scheduling for the formulation which is selected can be determined by standard procedures, well known in the art. Such procedures involve extrapolating an estimated dosing schedule from animal models, and then determining the optimal dosage in a human clinical dose ranging study.

Examples of molecules of the invention are set forth below.

Making and Using Agonistic Monoclonal Antibodies

A. Materials and Methods

In the Examples set forth below, the following procedures were used, as indicated in the examples.

Cell lines and culture conditions

The EBV-transformed B-cell line JY and the myeloid derived cell line THP1 were cultured in T75 culture flasks routinely in Iscove's modified Dulbecco's medium (IMDM) to which 50 □g/ml gentamycin and 2% heat inactivated foetal calf serum was added (FCSi; BioWhittaker, Verviers, Belgium). The cells were cultured in a humidified incubator at 37°C and 5% CO_2. Once or twice per week the cells were split (1/20 to 1/100). To store the cell line, ampoules were made containing 5-10 x 10^6 cells/ml Hank's balanced salt solution HBSS supplemented with 20% FCSi and 10% DMSO, and stored in the liquid nitrogen.

Peripheral mononuclear blood cell isolation and storage

Peripheral mononuclear blood cells (PBMC) were isolated from "buffy coats" of healthy donors by Lymphoprep™ (1.077 g/ml) density centrifugation and resuspended in Ca^{2+}/Mg^{2+}-free PBS-0.1% BSA. Autologous PBMC were stored in RPMI 1640 supplemented with 2 mM L-glutamine, 10% FCSi, 50 μg/ml gentamycin and 10% DMSO at –196 °C (for CD8 T cell purification, see below).

Monocyte enrichment and generation of monocyte-drived immature DC

Monocytes were purified from PBMC by immunomagnetic depletion (monocyte-enrichment cocktail containing Mabs against CD2, CD3, CD16, CD19, CD56, CD66b and glycophorin A; StemSep™ from StemCell Technologies, Vancouver, Canada). Monocyte (>90% $CD14^+$) preparations devoid of neutrophilic granulocytes, platelets, lymphocytes and NK cells were subsequently cultured in serum-free culture medium, StemSpan™ (StemCell Technologies), supplemented with 10 ng/ml GM-CSF and 20 ng/ml IL-4 (both cytokines from

PeproTech, Rocky Hill, NJ, USA) at 37°C /5%CO_2 during 6-7 days. These monocytes were seeded at a cell density of $1x10^6$/2ml/10 cm^2 polystyrene surface (coated with 12 mg/ml/ 10 cm^2 poly-hydroxethyl-methacrylate; Sigma) and fresh GM-CSF/IL-4 was added at day 2 and 5. After 6-7 days, the nonadherent cells (with a dendritic morphology) were collected and displayed the following (flow cytometry, see below) phenotypic profile: $CD1a^+$, $CD14^-$, $CD40^+$, $C80^+$, $CD83^-$, $CD86^+$, $HLA-DR^+$ and mannose $receptor^{++}$.

CD8 T lymphocyte isolation

Autologous PBMC were thawed, and CD8 T lymphocytes were purified by immunomagnetic depletion of other cell types (CD8-enrichment cocktail with Mabs against CD4, CD14, CD16, CD56 and glycophorin A; StemCell Technologies). This procedure resulted in >90% $CD3^+/CD8^+$ lymphocytes devoid of monocytes, neutrophilic granulocytes, platelets, B and CD4 lymphocytes, and NK cells.

Flow cytometric analyses

Cells ($0.1x10^6$ cells/100 µl PBS-0.1% BSA/sample) were incubated with conjugated (to either fluorescein isothiocyanate, phycoerythrin or peridinin chlorophyll protein) Mabs (Becton & Dickinson, Woerden, The Netherlands) for 15 min. at 21°C, and then thoroughly washed in PBS-0.1% BSA and analyzed on a flow cytometer (FACSCalibur™; Becton & Dickinson, Woerden, The Netherlands).

Competition of CD40 ligand and anti-CD40 monoclonal antibodies on JY cells

Blocking of soluble CD40 ligand (sCD40L) binding by anti-CD40 monoclonal antibodies (Mabs) was demonstrated by using JY cells, which express high levels of CD40. These cells ($0.1x10^6$ cells/100 µl PBS-0.1% BSA/sample) were pre-incubated with anti-CD40 Mabs for

15 min. at 21°C, and then thoroughly washed in PBS-0.1% BSA, followed by an incubation with a soluble fusion protein consisting of the extracellular domain of human CD40L fused to the extracellular domain of murine CD8α (CD40L-mCD8α; Kordia, Leiden, The Netherlands and Tanox Pharma BV, Amsterdam, The Netherlands) for 15 min. at 21°C. Subsequently, CD40L-mCD8α was detected by using rat anti-mouseCD8α coupled to phycoerythrin, and analyzed by flow cytometry.

Well-characterized control Mabs were included as controls: M2 and G28-5 compete for the CD40L binding site, and 5C3 binds to a region distinct from the CD40L binding site. EA-5 partially inhibits the binding of CD40L to its receptor (Pound et al., Int Immunol 1999, 11, p11-20).

Inhibition of binding of anti-CD40 monoclonal antibodies to membrane CD40L by CD40-Fc (IgG4).

As a source of membrane CD40L activated CD4+ T cells are used. To this purpose expression of CD40L on T cells is induced through culturing plastic non-adherent PBMC with PMA and ionomycine for 6 hrs in IMDM + 5% human pooled AB serum. CD40-Fc (IgG4 made by Tanox Inc Houston USA) is directly added at a saturating dose to the activated T cells or after pre-incubation of CD40-Fc with excess of anti-CD40 Mabs. Binding of CD40-Fc to CD40L activated CD4+ (CD3+CD8-) T cells is monitored through FACS analysis after staining with PE conjugated goat anti human IgG-Fc, FITC conjugated CD3 and PERCP conjugated CD8.

CD40 ELISA

ELISA plates (Immunon 2) were coated overnight at room temperature with 0.5 µg/ml, 50 µl per well of goat-anti-human IgG (Fc). Next the plates were treated with 1% BLOTTO for 60 min at room

temperature. After washing 4 times with PBS/Tween, 50 µl/well of CD40-Fc plus 50 µl of supernatants of the fusion wells were added and incubated for 1 hour at room temperature. After another 4 washings with PBS/Tween, 50 µl of goat-anti-mouse IgG (Fc)-HRP conjugate was added and incubated for 1 hour. After 4 washings the substrate TMB was added at 100 µl/well to the plates which were incubated for 30 min. The reaction was stopped by addition of 50 µl/well of 0.2 M H_2SO_4 and the plates were read with an ELISA reader at 450/590 nm

THP-1 assay

Stimulation of THP-1 cells

3 x 10^6 THP-1 cells were first cultured for two days in 10 ml of IMDM + 2% of human type AB serum in the presence of 5 x 10^2 U/ml IFN-□. Next the IFN-g treated THP-1 cells were washed once in IMDM + 2% human type AB serum. 10^4 THP-1 cells per 96 w plate well were cultured for two days in 120 µl of culture medium diluted 1:2 with hybridoma supernatant. As controls CD154-mCD8 was used at 40 □g/ml maximum and 2x dilutions and LPS at 20 ng/ml maximum and 2x dilutions.

Measurement of IL-8

ELISA plates were coated with mouse anti human IL-8 antibody (Serotec) at 5 µg/ml, 100 □l/well for 2 hrs at room temperature on a plate shaker. The plates were then incubated with 1% BLOTTO for one hour on the plate shaker at room temperature. After four washings with PBS/Tween, 80 µl of supernatants harvested from the THP-1 plate were added to the ELISA plate. For the IL-8 standards: IL-8 was diluted with 1% BLOTTO to 1000 pg/ml, 300 pg/ml, 100 g/ml 30 pg/ml, 10 pg/ml, 3 pg/ml, 1pg/ml. The ELISA plates were incubated for one hr at room temperature on the plate shaker. After four washings with PBS/Tween,

100 µl/well mouse-anti IL-8 biotin conjugate (Serotec) was added at 1:1000 dilution in 1% BLOTTO and the plates were incubated for one hour at room temperature. After four washings with PBS/Tween, 100 □l/well AMDX SA-HRP at 1:1000 dilution in 1% BLOTTO was added to the wells and the plates were incubated for 1 hour at room temperature on the plate shaker. After 4 washings with PBS/Tween, 100 □l of TMB substrate was added to each well and the plates were incubated for 30 minutes at room temperature on the plate shaker. The reaction was stopped by addition of 50 µl/well of 0.2 M H_2SO_4 and the plates were read with an ELISA reader at 450/590 nm.

Induction of mature DC

Immature DC (see above) are cultured in the presence of anti-CD40 Mabs under serum-free condition (StemSpan™) at 37°C /5%CO_2 for 48 hours. In addition, CD40L-mCD8α, LPS and a combination of IL-1β and TNF-α are used as well-established controls for DC maturation. The change from immature to mature DC is determined by: (1) phenotype ($CD1a^+$, $CD14^-$, $CD40^{+++}$, $CD80^{+++}$, $CD83^+$, $CD86^{+++}$, $HLA\text{-}DR^{+++}$, mannose receptor$^-$), (2) IL-12p70 production (commercially available kit), and by (3) the capability of inducing influenza-matrix peptide specific autologous cytotoxic $CD8^+$ T lymphocytes (see below).

IL-12p70 ELISA

Immature DC are cultured in the presence of anti-CD40 Mabs (1µg/ml) with or without IFN-g (1000 U/ml) for 48 hrs. IL-12p70 secretion was measured in the supernatant using a commercially available kit from Diaclone Research, Becanson, France. Inhibition of IL-12 production was obtained by preincubation of anti-CD40 Mabs with 10 times excess of CD40-Fc (IgG4 made by Tanox Inc Houston USA) for 15 min at room temperature.

Induction of cytotoxic $CD8^+$ T lymphocytes by mature DC

Mature DC generated by agonistic anti-CD40 Mabs are loaded with a synthetic influenza matrix peptide (Flu-peptide $_{58-66}$); $1x10^6$ DC/Flu-peptide 5 μg/ml StemSpan™) and co-cultured with $0.5x10^6$ purified autologous CD8+ T lymphocytes at 37°C /5%CO_2 during 7 days. Cytotoxicity of the CD8+ T lymphocytes is determined by: (1) enumeration of the number of IFN-γ producing T cells, which are representative for activated CTL (using flow cytometry with an IFN-γ detection kit from Miltenyi Biotec, Bergisch Gladbach, Germany), and (2) a convential assay measuring cytolysis by CTL of target cells loaded with Flu-peptide.

Examples

Example 1: Generation of mouse anti-human CD40 monoclonal antibodies

Two immunization protocols were used to generate anti-CD40 monoclonal antibodies. In the first, female BALB/c mice were injected intraperitoneally with SF-9 cells expressing CD40 (3×10^6 cells/mouse), which were washed with PBS twice before injection. At day 17 and 31 the mice received a booster injection with SF-9 cells. Fourteen days after the last the last the injection the spleen cells were isolated and 10^8 cells were used for cell fusion with 10^8 SP2/0 murine myeloma cells using polyethylene glycol. The fused cells were re-suspended in D15 (a modified DMEM medium) supplemented with HAT, followed by plating on fifty-one 96 wells plates. After 10 - 14 days supernatants of wells containing growing hybridoma cells were screened for anti-CD40 antibody production in an ELISA. This analysis showed that a total of 69 wells out of 4896 seeded wells contained hybridomas

producing anti-human CD40 specific antibodies. Culture supernatants of these wells were selected for additional experiments, such as for studying the induction of IL-8 secretion from THP-1 cells (see below). Next limiting dilution was performed twice to obtain clones from a number of hybridoma lines that produced CD40 agonist antibodies. For this purpose hybridoma cells were seeded at densities of less than 1 c/well in 96 well plates and cultured for 3-4 weeks. Supernatant of positive wells was screened in the CD40 ELISA and the THP-1 assay for the presence of CD40 binding antibodies.

For the second immunization regimen, BALB/c mice were injected intraperitoneally with 2.5 x 10^6 monocyte-derived immature DC. At days 14, 35 and 55 mice received booster injections with monocyte-derived DC from different donors. At around day 100-120, spleen cells will be isolated and fused with murine myeloma cells in analogy to the above protocol. Supernatants of wells with growing hybridomas will be screened for the presence of CD40 binding antibodies in the ELISA. Hybridoma supernatants containing CD40 binding antibodies will be subsequently screened for potential agonistic activity as described for the hybridoma's originating from B cells isolated from the BALB/c immunized with CD40 expressing SF-9 cells

Example 2

Screening the CD40 binding antibody samples from hybridoma lines for agonistic activity on THP-1 cells and subsequent cloning of lines and testing of monoclonal mabs

To screen for antibodies with agonistic activity, the selected supernatants containing CD40 binding antibodies were subsequently tested for their ability to induce IL-8 production in the CD40 expressing

monocytic cell line THP-1, which had been pre-incubated with IFN-γ. As shown in table 1, most supernatants contained anti-CD40 antibodies, which displayed agonistic activity in this assay. Supernatants were arbitrarily subdivided into four different groups on the basis of their performance in the THP-1 assay (strong agonists with an OD of >2.000, intermediate agonists with an OD between 1.000-2.000, low agonists with an OD between 0.375-0.999 and non-agonists with an OD <0.375).

A number of the hybridoma lines were cloned and monoclonal antibodies from the resulting clones were also tested in the THP-1 assay. Most but not all clones retained the reactivity pattern of the corresponding mother lines (data not shown).

Example 3

Assaying the ability of the CD40 reactive antibody clones to drive maturation, IL-12p70 production and priming for CTL activation of immature DC

DC derived from monocytes after culture with GM-CSF and IL-4 represent immature DC. Anti-CD40 monoclonal antibodies have been assayed for their capacity to induce maturation of these CD40 expressing immature DC. Experiments from other investigators have shown that stimulation of monocyte-derived DC with sCD40L results in their differentiation into DC with a mature phenotype. Furthermore, sCD40L in combination with IFN-γ stimulates monocyte-derived DC to secrete IL-12p70. In contrast to immature DC, mature DC express CD83. In addition, compared to immature DC, mature DC display enhanced expression on a per cell basis of the co-stimulatory molecules CD80 and CD86, decreased expression of the mannose receptor and

loss of the ability to efficiently take up molecules, as shown for dextran-FITC. At first the phenotypical changes that accompany the differentiation of immature to mature DC were monitored by FACS-analysis as a read-out for induction of DC maturation by the anti-CD40 monoclonal antibodies. Antibodies were first used on their own to stimulate monocyte-derived DC. As shown in fig 1 (combined results of several experiments showing CD83 up-regulation and mannose receptor down-regulation) and in fig. 2 (one typical experiment showing induced expression of CD80, CD83 and CD86) CD40 binding antibodies were tested and were found to induce phenotypical maturation of monocyte derived DC, as is indicated by the increased percentage of cells expressing the CD83 marker, the increased expression on a per cell basis (mean fluorescence intensity; MFI) of CD80 and CD86 and decreased expression of the mannose receptor. Remarkably, some of the clones that did not induce IL-8 production in THP-1 cells could induce maturation of DC, demonstrating that agonist properties of CD40 monoclonal antibodies may differ between different CD40 expressing cell types (data not shown).

In addition, the IL-12p70 production of monocyte derived DC was tested after stimulation with the CD40 monoclonal antibodies and IFN-γ, since dendritic cells require stimulation through at least two different pathways to produce IL-12p70 (Kalinski et al Blood 1997 90:1926). Our results show that apart from induction of phenotypical maturation, the CD40 agonist antibodies also induced IL-12 production in DC when used together with IFN-γ (fig 3). Our finding that pre-incubation of the CD40 monoclonal antibodies with excess of CD40-Fc inhibited induction of IL-12 production demonstrated that the agonistic effect of the antibodies is

really exerted through CD40 and not through other membrane expressed molecules on the DC (fig 4).

In the mouse, T cell help to CTL was found to be mediated through CD40 activated DC. Antigen dependent interaction of helper T cells with DC did not only result in the activation of the helper T cell, but through CD40L-CD40 interaction also in the activation of the DC. Only in their activated stage DC were able to prime CTL responses. In the absence of helper T cells no DC activation and therefore no CTL priming occurred. However, by means of in vivo administration of an anti-mouse CD40 stimulatory antibody, T cell help could be efficiently bypassed and DC directly activated.

To show that the same mechanism of CTL activation applies to man, an *in vitro* study was performed in which CTL activation was studied in a co-culture system consisting of purified human $CD8^+$ T cells, monocyte-derived DC as APC and a minimal peptide derived from influenza virus matrix protein as antigen. This peptide constitutes a dominant HLA-A2 restricted CTL epitope. This experiment was carried out to establish whether our anti-CD40 monoclonal antibodies could empower monocyte-derived DC with an increased ability to stimulate CTL responses compared to untreated control DC. CTL activation was analyzed in this experiment through measurement of production of IFN-γ by activated CTL and enumeration of expansion of CTL with PE conjugated HLA-A2/influenza matrix peptide tetramers. As shown in fig 5a and b the stimulation of monocyte derived DC with CD40 monoclonal antibodies led to increased ability of these cells to induce a flu peptide directed CD8+ T cell response. For most antibodies this effect was elevated when, in addition to the monoclonal antibodies, IFN-γ was used in the pre-activation of the dendritic cells.

Example 4

Analysis of the inhibition of the binding of sCD40L to CD40 by the anti-CD40 antibody samples

Anti-CD40 antibodies that synergize with sCD40L in the induction of CD40 mediated activation of DC most likely show co-binding with sCD40L to CD40 and thus do not display strong blocking of binding of sCD40L to CD40. To screen for such antibodies, the percentage of inhibition of sCD40L binding to CD40 on JY EBV transformed B cells by the monoclonal antibodies was tested. This analysis revealed that there was strong variation in the degree that the monoclonal antibodies could inhibit the binding of sCD40L to CD40. Some antibody samples almost completely inhibited sCD40L binding, whereas other antibody samples could only partially block sCD40L binding or had no effect at all (table 2). The results were confirmed in the reverse way for a limited number of clones by testing the inhibition caused by the anti-CD40 monoclonal antibodies of the binding of CD40-Fc to CD40L expressed on the membrane of PMA + ionomycine activated CD4+ T cells. In this experiment clone 4 blocked binding of CD40-Fc to CD40L on the T cells for 88%, clone 7 and 64 for respectively 16% and 25%. Although there was no absolute correlation between the performance of the antibodies in the DC maturation and the THP-1 assay and their ability to block sCD40L binding to CD40, all the clones that did not block this interaction were non-responders in both assays (data not shown)

Example 5

Synergism between anti-CD40 antibodies and mCD40L or sCD40L in agonist activity on DC

It is predicted that those antibodies that to a major extent block binding of sCD40L to CD40 will not display synergism with sCD40L in the induction of DC maturation or other agonistic properties exerted on CD40 positive cells. In contrast, some of the CD40 binding antibodies that efficiently co-bind with sCD40L to its receptor will presumably show synergism with sCD40L or membrane bound CD40L (mCD40L) in driving DC maturation. (As a source of membrane bound CD40L antigen or mitogen activated CD4+ T cells will be used) This will be demonstrated by the increased percentage of cells expressing CD83, by the increased expression on a per cell basis of CD80 and CD86 and the decreased expression of the mannose receptor. Also the level of IL-12p70 produced by the DC after stimulation by the combination of one of these antibodies with sCD40L and IFN-γ will be enhanced compared to the level induced by sCD40L and IFN-γ alone. Apart from synergism between sCD40L and an anti-CD40 antibody, two anti-CD40 antibodies may also show synergism with each other in the induction of IL-12p70 secretion. This synergism may occur most noticeably between antibodies that block binding of sCD40L to CD40 and those that are partial or non-inhibitors of this interaction, as these antibodies are expected to bind different epitopes on CD40.

In analogy to the experiment in which the maturation of DC was tested, the effect on CTL activation of the anti-CD40 antibody samples, used on their own or together with sCD40L, will be evaluated in future experiments. It is expected that, resulting from more efficient stimulation

of the DC, synergism in CTL activation will occur between the same combinations of sCD40L and monoclonal antibodies as in the maturation assay. The same holds true with regard to synergism in CTL activation between two different anti-CD40 antibodies.

Example 6

Enhanced potency, in comparison to CD40 agonist antibody, of a bispecific antibody directed to CD40 and 4-1BB ligand or a bispecific antibody directed to CD40 and CD28 in the ability to license DC for CTL activation

Use of a bi-specific antibody with specificity for CD40 on one side and a determinant on T cells on the other side potentially has the benefit of bringing the activated DC in close contact with surrounding T cells. If the antibody part that recognizes the T cell determinant has agonistic properties, the additional benefit may be that the attracted T cell will be stimulated both through the signals delivered by the activated DC and the agonistic properties of the T cell part of the bi-specific antibody. This possibility will be evaluated by comparing the effect of the addition of the CD40 monoclonal antibodies and the bi-specific antibodies in the above described DC-CTL co-culture system, using flu peptide specific CD8+ T cell responses as read out.

The description and examples are exemplarily only and not limiting, and the invention is defined only by the claims which follow, and includes all equivalents, known and unknown, of such claimed subject matter.

What Is Claimed Is:

1. Agonist anti-CD40 molecules that can bind to and stimulate professional or non-professional human APCs.
2. The agonist anti-CD40 molecules of claim 1 wherein the human professional APCs are human dendritic cells
3. Agonist anti-CD40 molecules which enhance the stimulatory effect of CD40L on CD40 positive cells.
4. Agonistic anti-CD40 molecules which induce phenotypical and functional maturation of monocyte derived dendritic cells.
5. The agonist anti-CD40 molecules of claim 1, 2, 3 or 4 wherein said molecules can simultaneously bind to CD40 with CD40L, as determined by their inability to inhibit CD40L binding to CD40 or their inability to inhibit of binding of sCD40 to CD40L..
6. The agonist anti-CD40 molecules of claim 1, 2, 3 or 4 which completely inhibit CD40L binding to CD40.
7. A composition comprising a combination of agonist anti-CD40 molecules according to any of claims 1 to 5.
8. The composition of claim 7 including at least one agonist anti-CD40 molecule which is a strong activator of binding of sCD40L to CD40, and at least one agonist anti-CD40 molecule which is a partial or non-inhibitor of binding of sCD40L to CD40 and of binding of sCD40 to CD40L.
9. The agonist anti-CD40 molecules of any of claims 1 to 6 which are monoclonal antibodies.
10. A bispecific agonist anti-CD40 monoclonal antibody wherein each specificity is for a different epitope on CD40.

11. The bispecific monoclonal antibody of claim 10 wherein one specificity is a strong activator of binding of sCD40L to CD40, and one specificity is a partial or non-inhibitor of binding of sCD40L to CD40.

12. The monoclonal antibodies of claim 9 which are chimeric, humanized, Deimmunised™ or human.

13. The monoclonal antibodies of claim 12 or fragments, analogues or homologues thereof, or a peptide, oligonucleotide, peptidomimetic or an organic compound which binds to the same epitopes as such antibodies.

14. The fragments of claim 13 which are Fab, $F(ab')_2$ Fv or single chain Fv.

15. Cell lines producing the monoclonal antibodies or fragment thereof of claim 9.

16. Cell lines producing the monoclonal antibodies or fragment thereof of claim 12.

17. Gene constructs coding for any of the molecules of claim 12.

18. Gene constructs coding for any of the molecules of claim 13.

19. Cells transfected or infected with the gene constructs of claim 17.

20. Cells transfected or infected with the gene constructs of claim 18.

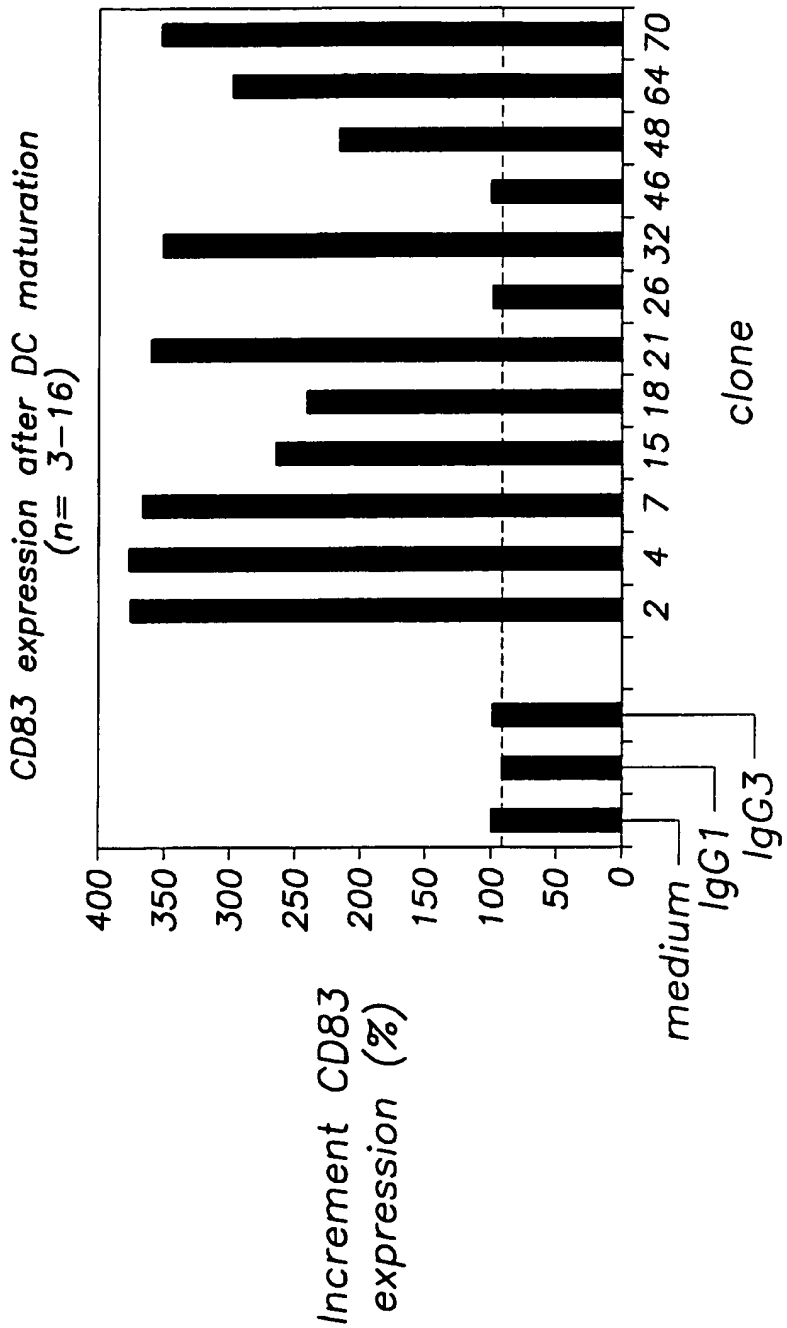

CD83 expression after DC maturation
(n= 3–16)
Increment CD83
expression (%)
400
350
300
250
200
150
100
50
0
2 4 7 15 18 21 26 32 46 48 64 70
clone
medium
IgG1
IgG3

FIG. 1a

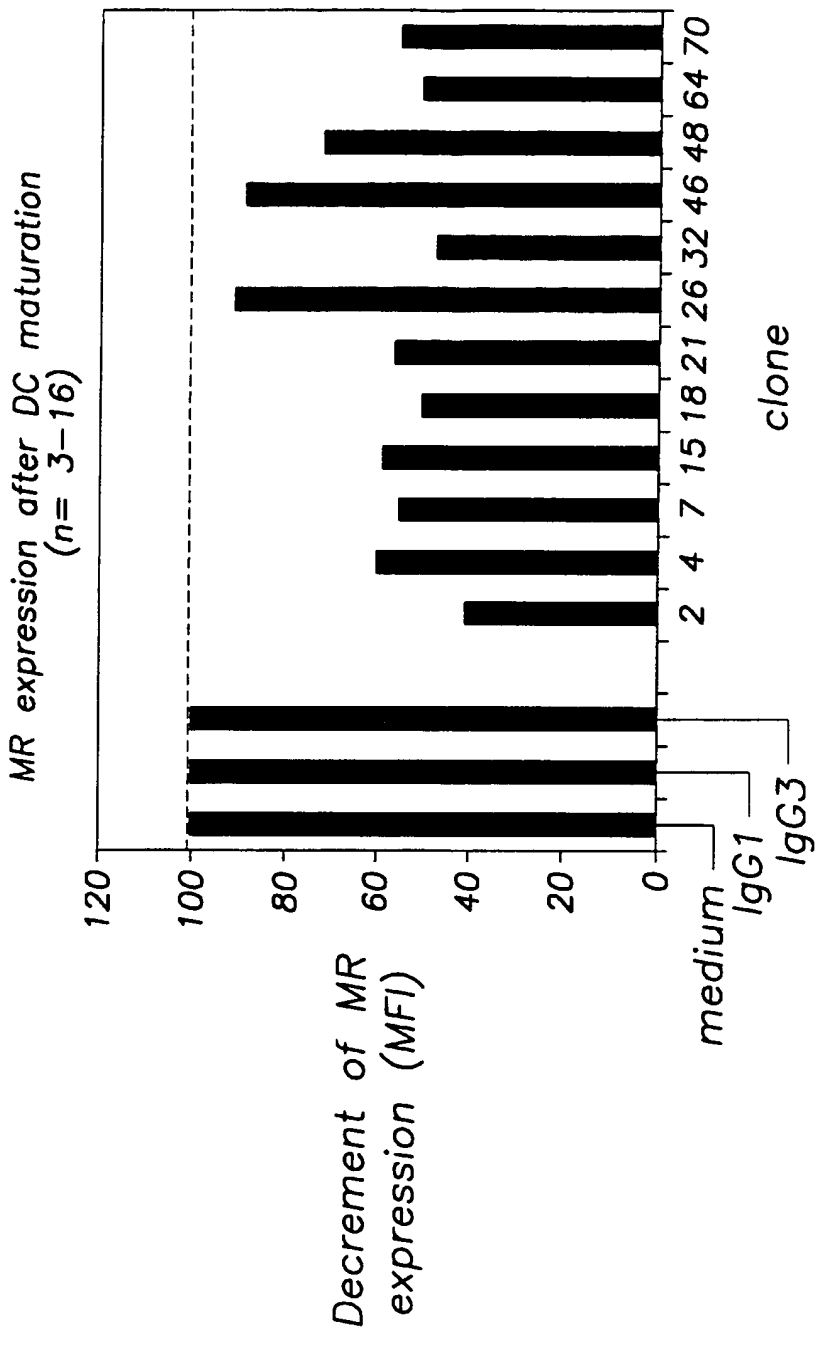

MR expression after DC maturation
(n= 3–16)
Decrement of MR
expression (MFI)
120
100
80
60
40
20
0
medium
IgG1
IgG3
2 4 7 15 18 21 26 32 46 48 64 70
clone

FIG. 1b

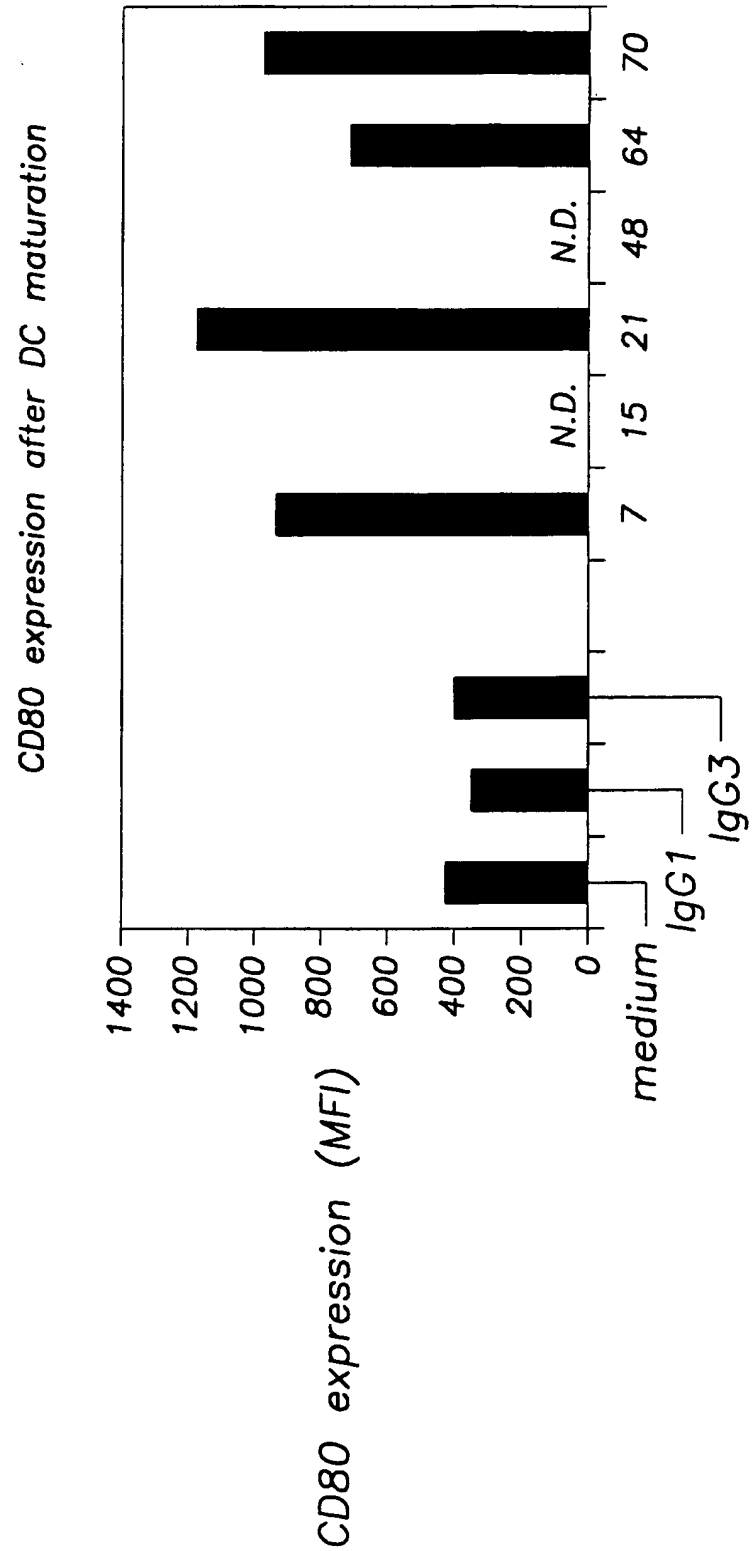

CD80 expression after DC maturation
CD80 expression (MFI)
1400
1200
1000
800
600
400
200
0
medium
IgG1
IgG3
7
15
21
48
64
70
N.D.
N.D.

FIG.2a

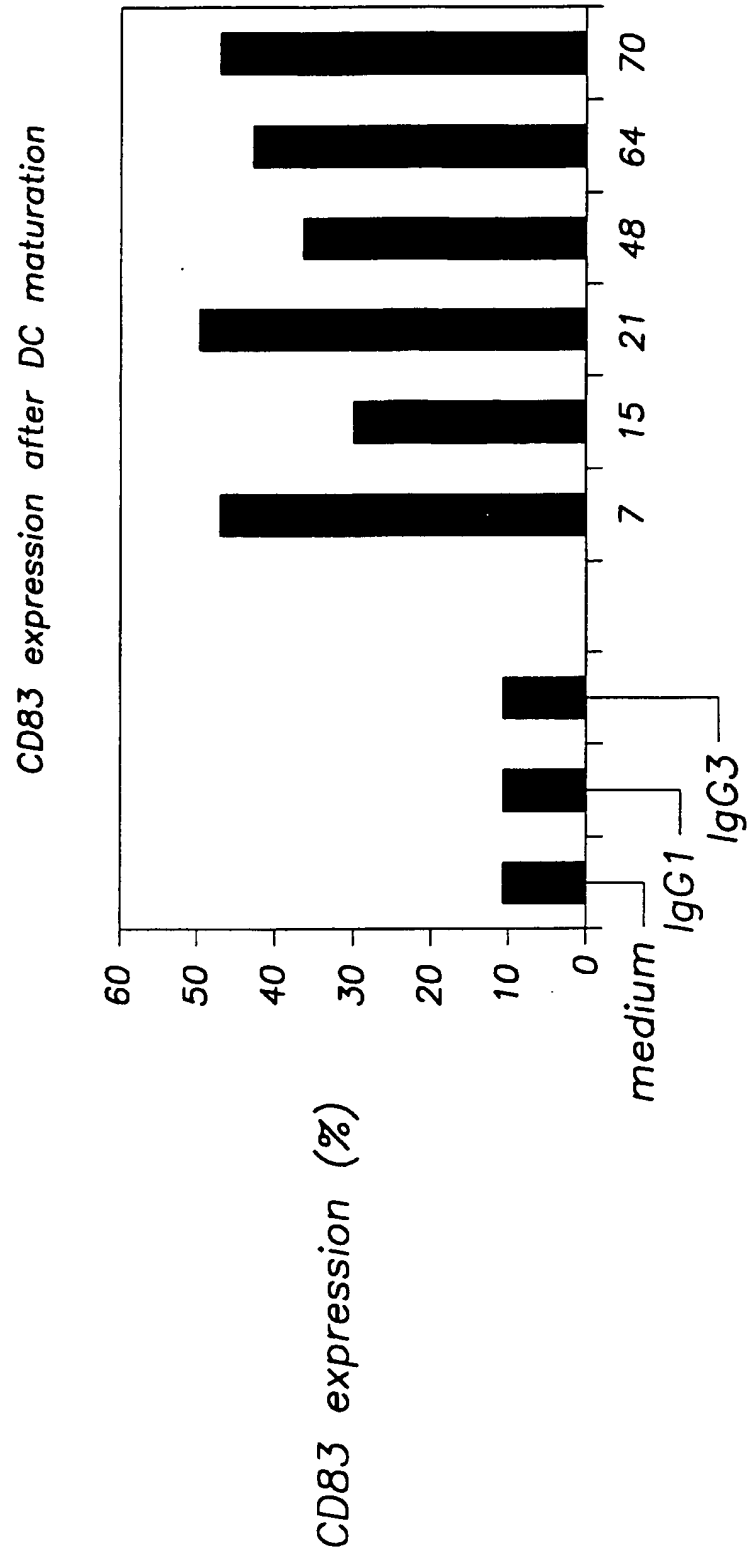

CD83 expression after DC maturation
CD83 expression (%)
60
50
40
30
20
10
0
medium
IgG1
IgG3
7
15
21
48
64
70

FIG.2b

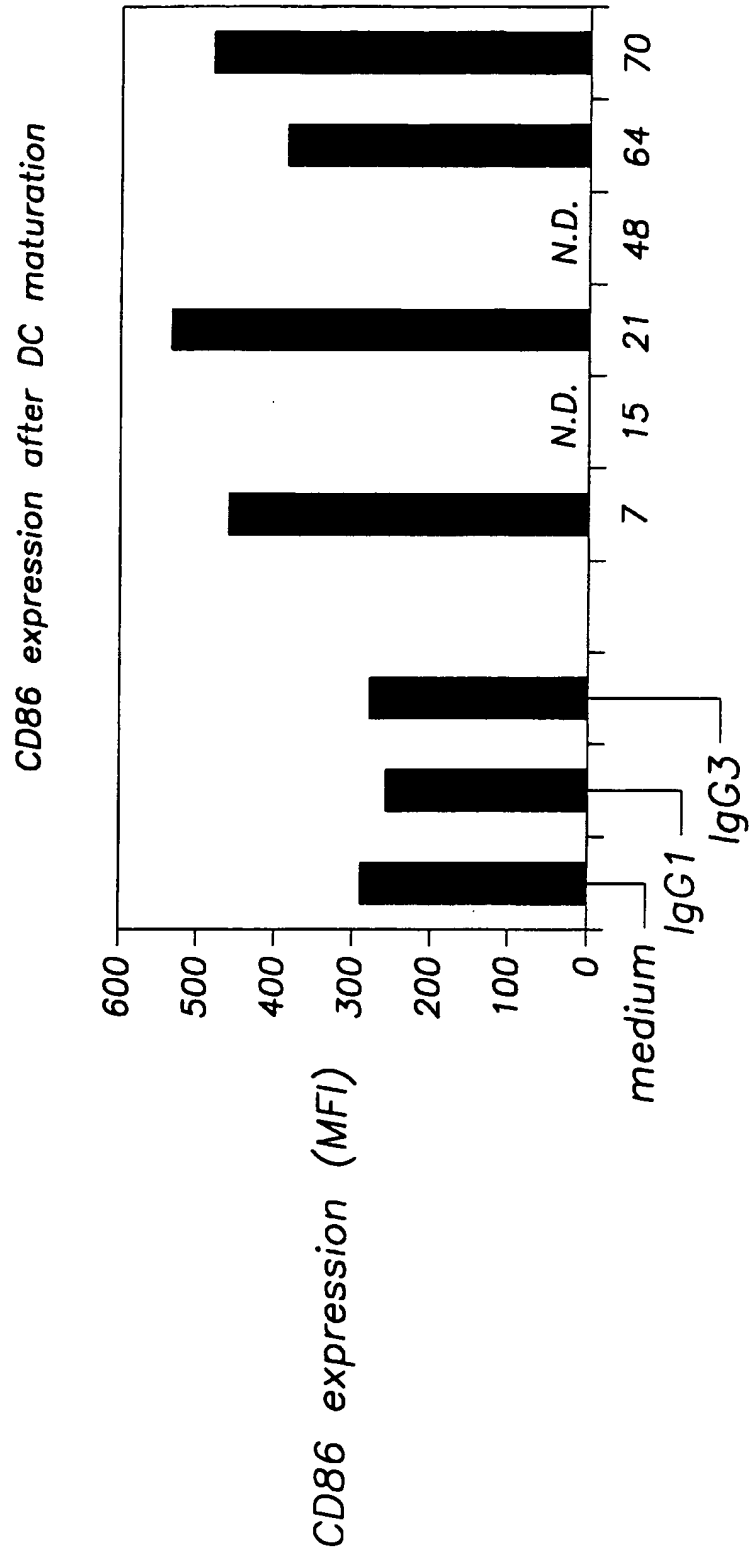

CD86 expression after DC maturation
CD86 expression (MFI)
600
500
400
300
200
100
0
medium
IgG1
IgG3
7
15
21
48
64
70
N.D.
N.D.

FIG.2c

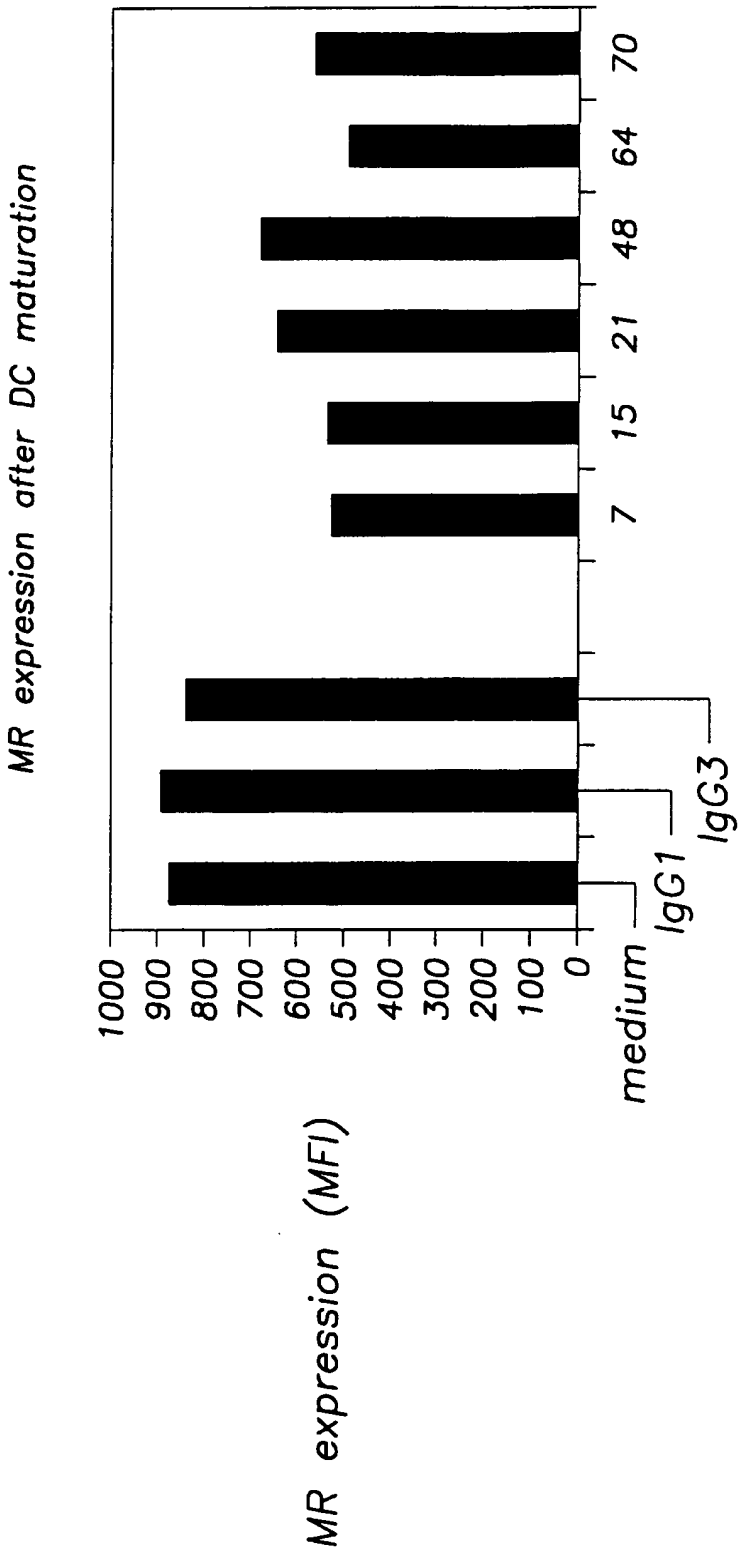

MR expression after DC maturation
MR expression (MFI)
1000
900
800
700
600
500
400
300
200
100
0
medium
IgG1
IgG3
7
15
21
48
64
70

FIG.2d

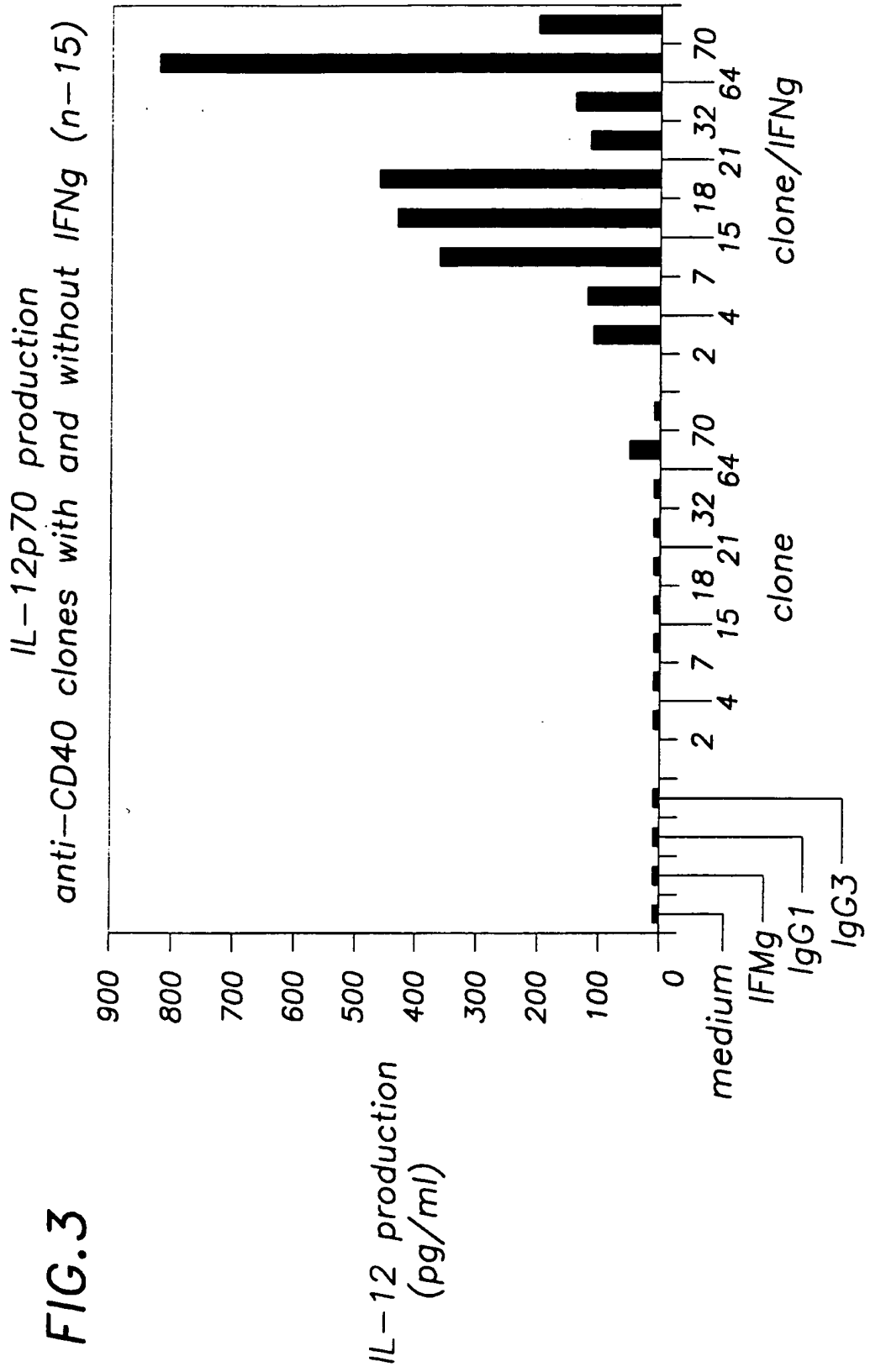
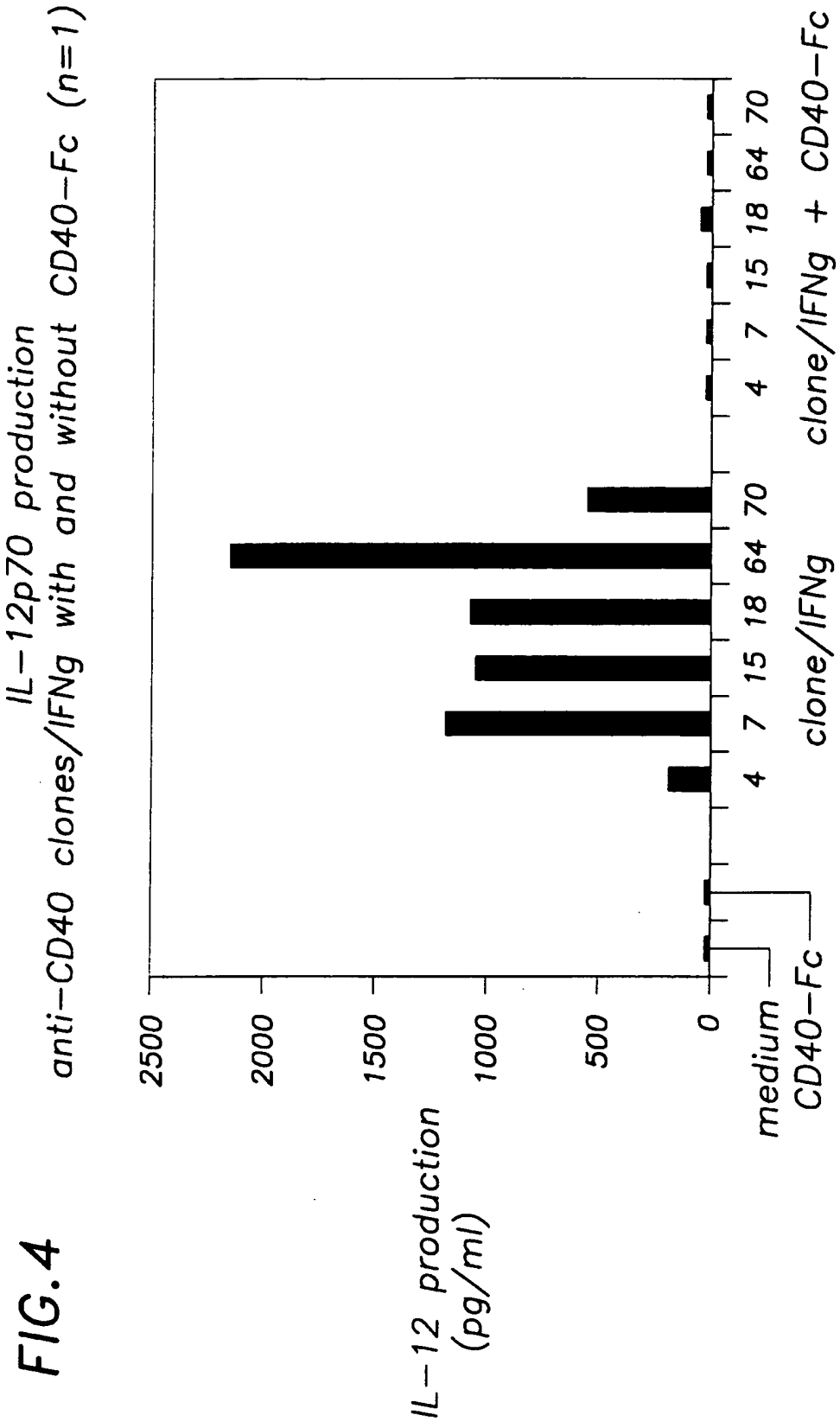

IL-12p70 production
anti-CD40 clones with and without IFNg (n-15)
IL-12 production (pg/ml)
900
800
700
600
500
400
300
200
100
0
medium
IFMg
IgG1
IgG3
2
4
7
15
18
21
32
64
70
clone
2
4
7
15
18
21
32
64
70
clone/IFNg

FIG.3

FIG. 4


IL-12p70 production
anti-CD40 clones/IFNg with and without CD40-Fc (n=1)
IL-12 production (pg/ml)
2500
2000
1500
1000
500
0
medium
CD40-Fc
4 7 15 18 64 70
clone/IFNg
4 7 15 18 64 70
clone/IFNg + CD40-Fc

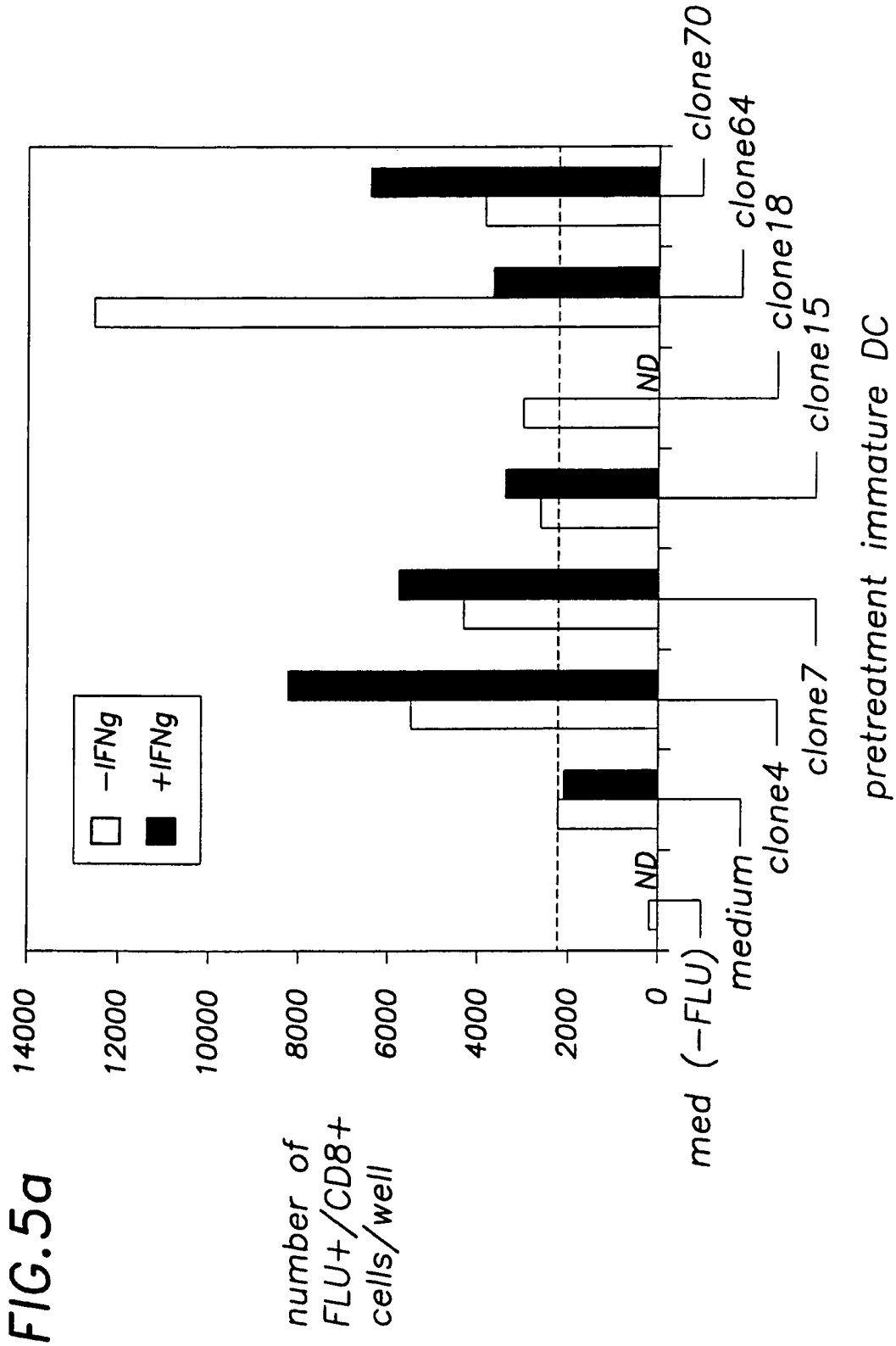

FLU-specific CD8 cells after 7 days co-culture
number of FLU+/CD8+ cells/well
14000
12000
10000
8000
6000
4000
2000
0
-IFNg
+IFNg
med (-FLU)
medium
ND
clone4
clone7
clone15
clone18
clone64
clone70
pretreatment immature DC

FIG.5a

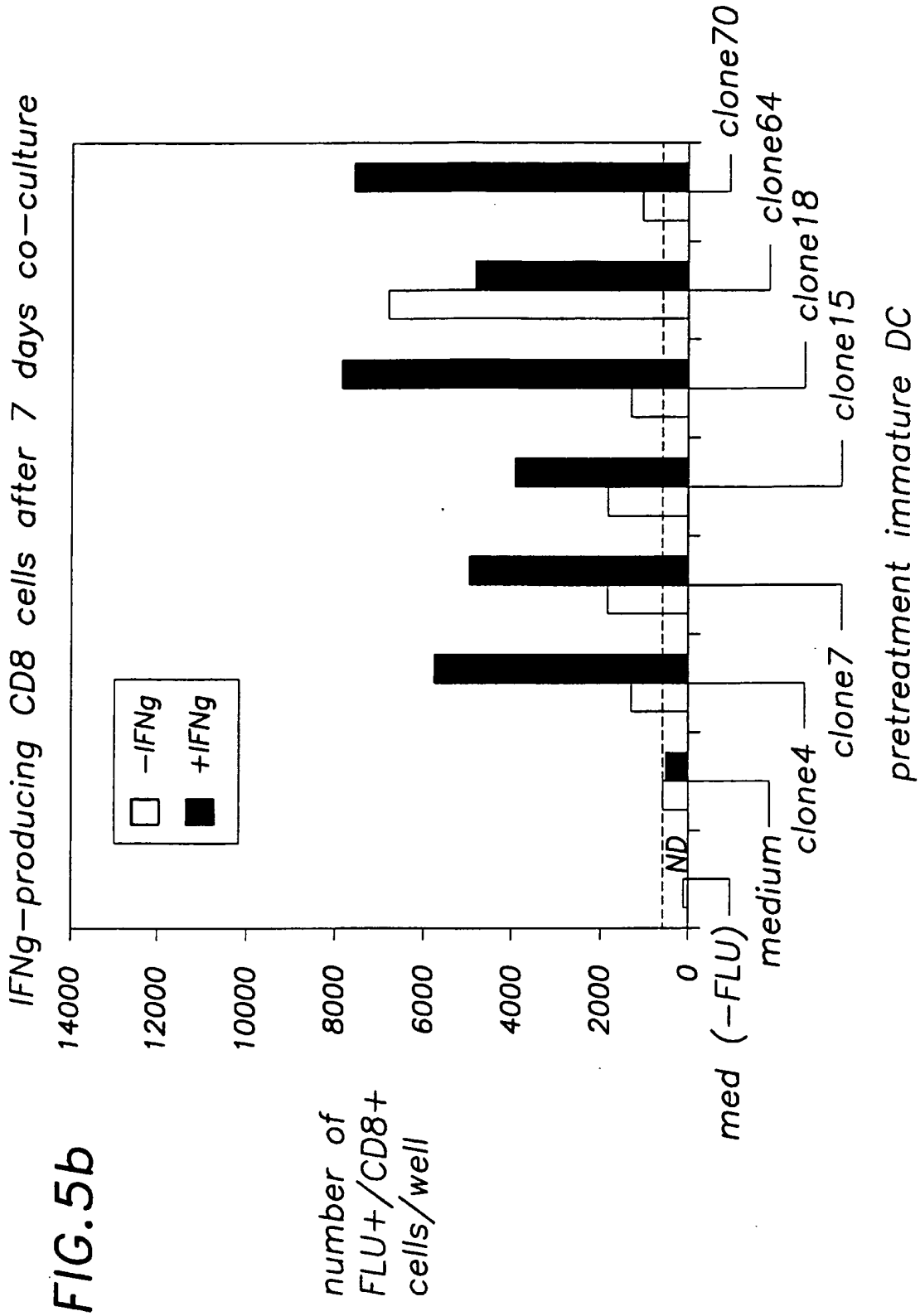

IFNg-producing CD8 cells after 7 days co-culture
number of FLU+/CD8+ cells/well
14000
12000
10000
8000
6000
4000
2000
0
-IFNg
+IFNg
ND
med (-FLU)
medium
clone4
clone7
clone15
clone18
clone64
clone70
pretreatment immature DC

FIG.5b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/03378

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIAGLOG, BIOSIS, CA, EMBASE, MEDLINE; WEST
search terms: cd40, antibod?, agonist?, bispecific

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	SCHWABE et al. Modulation of Soluble CD40 Ligand Bioactivity with Anti-CD40 Antibodies. Hybridoma. 1997, Volume 16, Number 3, see entire document.	1-6 ------- 1-6, 10-11
Y	US 5,534,254 A (HUSTON et al.) 09 July 1996, see entire document.	1-6, 10-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 MARCH 2001	Date of mailing of the international search report 23 MAY 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PHILLIP CAMBEL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/03378

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-9 and 12-20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/03378

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 39/395; C07K 16/00, 16/28; C12N 5/10, 5/12, 15/00, 15/11, 15/12, 15/13, 15/63

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/130.1, 133.1, 135.1, 136.1, 141.1, 143.1, 144.1, 153.1, 173.1 ; 435/451, 452, 445, 530/387.1, 387.3, 388.1, 388.2, 388.2 2, 388.7, 388.73; 536/23.1, 23.4, 23.5, 23.53

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

424/130.1, 133.1, 135.1, 136.1, 141.1, 143.1, 144.1, 153.1, 173.1 ; 435/451, 452, 445, 530/387.1, 387.3, 388.1, 388.2, 388.2 2, 388.7, 388.73; 536/23.1, 23.4, 23.5, 23.53

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(71) Applicant *(for all designated States except US)*: GENVEC, INC. [US/US]; 65 West Watkins Mill Road, Gaithersburg, MD 20878 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: ROELVINK, Petrus, W. [NL/US]; 17502 Gallagher Way, Olney, MD 20832 (US). KOVESDI, Imre [US/US]; 7713 Warbler Lane, Rockville, MD 20855 (US). BRUDER, Joseph, T. [US/US]; 10243 Royal St. Andrews Place, Ijamsville, MD 21754 (US). BROUGH, Douglas, E. [US/US]; 3900 Shallowbrook Lane, Olney, MD 20832 (US). MCVEY, Duncan, L. [US/US]; 6016 Muncaster Mill Road, Derwood, MD 20855 (US). WICKHAM, Thomas, J. [US/US]; 12031 Arista Manor Way, Germantown, MD 20876 (US).

(74) Agents: HEFNER, M., Daniel et al.; Leydig, Voit & Mayer, Ltd., Suite 4900, Two Prudential Plaza, 180 North Stetson, Chicago, IL 60601-6780 (US).

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/58478 A1

(54) Title: ANTIGENIC COMPLEXES AND METHODS

(57) Abstract: The present invention provides a complex that includes a virion having a ligand that recognizes an epitope present on an immune effector cell surface and at least a first nucleic acid encoding a first non-native antigen. The invention also provides a library including a plurality of such complexes, in which antigens of at least two of the plurality are different. Using such reagents, the invention provides a method of precipitating an immune response within an effector cell, wherein such a complex is delivered to the cell under conditions sufficient for the cell to mount an immune response to the antigen. When applied *in vivo*, the method can serve to immunize an animal from the pathogen. Moreover, using a library including a plurality of complexes, which contains at least one test antigen, the invention provides a method of assessing the antigenicity of the test antigen.

ANTIGENIC COMPLEXES AND METHODS

TECHNICAL FIELD OF THE INVENTION

The present invention pertains to antigenic complexes and methods of inoculating and immunizing animals.

BACKGROUND OF THE INVENTION

The identification of pathogenic organisms and viruses has led to the development of successful protocols for immunizing healthy individuals against such organisms. Early protocols involved exposing healthy individuals to live or attenuated pathogens to induce immune responses against the pathogens. With respect to many pathogens, such live or attenuated vaccines remain superior to other vaccines because of their tendency to elicit a broad level protective response. Other disorders, however, are caused or spread by pathogens less amenable to this approach. For example, it has proven difficult to develop or store live or attenuated vaccines derived from several common pathogens. Still other pathogens simply are not sufficiently antigenic to generate a sufficient (or even any) response in a host animal to be useful as a vaccine, whether through evolved stealthing defenses (e.g., HIV, herpes, etc.), limited presentation of antigen, genetic drift (e.g., influenza), or other proclivities. Of course, the possibility of actually causing the disease against which protection is intended (e.g., polio, measles, etc.) remains a major concern associated with this approach.

An alternative to the use of live/attenuated pathogen vaccines is to use antibodies raised against antigens associated only with an identified pathogen. Such approaches can be effective in some instances. However, no assurance can be had that any antibodies raised against a putative antigen will effectively protect against the pathogen providing the antigen. Thus, it is frequently necessary to test a large number of putative antigens isolated from a pathogen, rendering such approaches relatively costly and time consuming. Also, such approaches generally do not elicit the broad level of protective response associated with live vaccines.

Genetic or peptide immunization has emerged as an alternative to conventional vaccines. This technology involves inoculating DNA encoding a pathogen protein, or an isolated pathogen protein, into the host. Risk of infection is greatly reduced, and the DNA or protein vaccines can be delivered to cells not normally infected by the pathogen. Compared to conventional vaccines, the production of DNA or peptide vaccines is straightforward, and DNA and protein are considerably more stable than live/attenuated vaccines. However, some DNA

or protein vaccines are not effective against certain pathogens. Indeed, vaccine approaches that deliver entire proteins may direct the immune response against immunodominant epitopes only, and not against subdominant epitopes (e.g., EBV latent membrane protein). Alternatively, such approaches can direct an immune response against epitopes subject to antigenic variation, and in some instances, such approaches can actually result in tolerance to the pathogen (e.g., a tumor or a microbe), rather than immunity (see, e.g., Toes *et al.*, *Proc. Nat. Acad. Sci. (USA) 94*, 14660-65 (1997); Toes *et al.*, *Proc. Nat. Acad. Sci. (USA), 93*, 7855-60 (1996); Toes *et al.*, *J. Immunol.*, 156, 3911-18 (1996); Aichele *et al.*, *Proc. Nat. Acad. Sci. (USA), 91*, 444-48 (1994); Aichele *et al.*, *J. Exp. Med., 182*, 261-66 (1995)).

Other recent advances in vaccine technology have focused on the manner in which cellular immunity is acquired in the first instance. Recognition and destruction of at least some pathogens is performed principally by $CD8^+$ cytotoxic T lymphocytes (CTLs). The mounting of a CTL immune response requires that "foreign" proteins undergo intracellular processing to peptide fragments, a function performed with high efficiency by professional antigen presenting cells (APCs), such as B-cells, dendritic cells, lymphoid fibroblasts, Langerhans cells, macrophages, monocytes, peripheral blood fibrocytes, etc., and potentially other cells such as cortical thymus epithelial cells, Ia- Thy 1- cells, peritoneal exudate cells, and the like. The processed peptide fragments ultimately are presented at the cell surface complexed with major histocompatability complex (MHC) class I molecules, which constitute the first stimulatory signals recognized by a CTL.

Processing of antigens presented by class I MHC generally involves endogenous synthesis and cytoplasmic processing not involving endosomes. While, under some circumstances, exogenous antigens can enter the cytoplasm for processing by the nonendosomal pathway and presentation by class I MHC, typically, exogenous antigens are internalized and processed through endosomes for presentation by class II MHC. To exploit this biology, APCs pulsed with proteins or DNA have been employed in vaccines. Typically, dendritic cells are harvested and pulsed with the protein or DNA and then cultured to activate MHC-I or MHC-II responses. These cells can be used subsequently *in vivo* or *in vitro* to present the antigens to CTLs. While these approaches may prove promising, a matter of concern when using APCs as carriers is that isolating even limited amounts of such cells is labor-intensive. Moreover, MHC class II-deficient APCs, expressing only MHC-I often fail to induce protection (Schnell *et al.*, *J. Immunol., 164*(3), 1243-50 (2000)). This demonstrates the desirability of achieving both types of response.

In addition to the limitations of current vaccine technologies, the identification of suitably antigenic proteins, peptides, or other moieties from many species of pathogen remains elusive. Considering these, and other, drawbacks, a need remains for improved strategies for identifying suitable antigens and for using them to inoculate animals.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a complex that includes a virion having a ligand that recognizes an epitope present on an immune effector cell surface and at least a first nucleic acid encoding a first non-native antigen. The invention also provides a library including a plurality of such complexes, in which antigens of at least two of the plurality are different.

Such reagents are useful both in research and in the clinic. Thus, for example, the invention provides a method of precipitating an immune response within an immune effector cell, wherein such a complex is delivered to the cell under conditions sufficient for the cell to mount an immune response to the antigen. When applied *in vivo*, the method can serve to immunize an animal from the pathogen. Moreover, using a library including a plurality of complexes, which contains at least one test antigen, the invention provides a method of assessing the antigenicity of the test antigen. These and other aspects of the present invention, as well as additional inventive features, will be apparent upon reading the following detailed description.

DETAILED DESCRIPTION OF THE INVENTION

Within the inventive complex, the ligand on the virion typically (but need not be) proteinaceous. Examples of suitable ligands include (but are not limited to) short (e.g., about 6 amino acids or less) linear stretches of amino acids recognized by integrins, as well as polyamino acid sequences such as polylysine, polyarginine, etc. Inserting multiple lysines and/or arginines provides for recognition of heparin and DNA, and an RGD sequence can be used as a ligand to bind integrins, such as are present on immune effector cells. Tandem repeats of lysine, arginine, and/or histidine residues (e.g., three or more, five or more, or even as many as ten or more tandem lysine residues, tandem arginine residues, tandem histidine residues, or tandem mixtures of lysine or histidine) can be similarly employed. Other ligands can be specific for particular substrates, such as, for example, immunoglobulin-like molecules (e.g., FABs, ScABs, etc.), or known specific ligands (e.g., CD40L that recognizes the CD40 antigen). Thus, it will be

apparent that, in some embodiments, the ligand is native to the virion (i.e., being present in a wild-type virus from which the virion is derived), but in other embodiments the ligand is a non-native ligand.

The ligand can be any moiety that binds with relative specificity to a substrate present on the surface of an immune effector cell (e.g., a T-lymphocyte or a B-lymphocyte, a natural killer cell, a granulocyte, a macrophage, a monocyte, a polymorphonucleocyte, a dendritic cell. etc.). Such a cell surface binding site can be any suitable type of molecule, but typically is a protein (including a modified protein such as a glycoprotein, a mucoprotein. etc.), a carbohydrate, a proteoglycan, a lipid, a mucin molecule, or other similar molecule. Examples of potential cell surface binding sites include, but are not limited to, heparin and chondroitin sulfate moieties found on glycosaminoglycans; sialic acid moieties found on mucins, glycoproteins, and gangliosides; and common carbohydrate molecules found in membrane glycoproteins, including mannose, N-acetyl-galactosamine, N-acetyl-glucosamine, fucose, and galactose. As immune effector cells typically are classified by the presence of various cell-surface proteins, many suitable proteins are known in the art.

The inventive complex is useful for eliciting an immune response within a derived (i.e., target) immune effector cell, typically an APC. In many applications, dendritic cells are preferred target APCs because using the CD40 ligand (CD40L) as a targeting ligand also facilitates the priming of $CD8^+$ cells (see, e.g., Toes *et al.*, *Sem. Immunol., 10*, 443-48 (1998)), further enhancing the efficacy of the invention. Where dendritic cells are the desired target, the ligand can recognize a protein typically found on dendritic cell surfaces such as adhesion proteins, chemokine receptors, complement receptors, co-stimulation proteins, cytokine receptors, high level antigen presenting molecules, homing proteins, marker proteins, receptors for antigen uptake, signaling proteins, virus receptors, etc. Examples of such potential ligand-binding sites in dendritic cells include 2A1, 7-TM receptors, CD1, CD11a, CD11b, CD11c, CD21, CD24, CD32, CD4, CD40, CD44 variants, CD46, CD49d, CD50, CD54, CD58, CD64, ASGPR, CD80, CD83, CD86, E-cadherin, integrins, M342, MHC-I, MHC-II, MIDC-8, MMR, OX62, p200-MR6, p55, S100, TNF-R, etc. Preferably, where dendritic cells are targeted, the ligand recognizes the CD40 cell surface protein, such as, for example, a CD-40 (bi)specific antibody fragment or a domain derived from the CD40L polypeptide.

Where macrophages are the desired target, the ligand can recognize a protein typically found on macrophage cell surfaces, such as phosphatidylserine

receptors, vitronectin receptors, integrins, adhesion receptors, receptors involved in signal transduction and/or inflammation, markers, receptors for induction of cytokines, or receptors up-regulated upon challenge by pathogens, members of the group B scavenger receptor cysteine-rich (SRCR) superfamily, sialic acid binding receptors, members of the Fc receptor family, B7-1 and B7-2 surface molecules, lymphocyte receptors, leukocyte receptors, antigen presenting molecules, and the like. Examples of suitable macrophage surface target proteins include, but are not limited to, B7-1, B7-2, CD11c, CD13, CD16, CD163, CD1a, CD22, CD23, CD29, Cd32, CD33, CD36, CD44, CD45, CD49e, CD52, CD53, CD54, CD71, CD87, CD9, CD98, Ig receptors, Fc receptor proteins (e.g., subtypes of Fcα, Fcγ, Fcε, etc.), folate receptor β, HLA Class I, Sialoadhesin, siglec-5, and the toll-like receptor-2 (TLR2).

Where B-cells are the desired target, the ligand can recognize a protein typically found on B-cell surfaces, such as integrins and other adhesion molecules, complement receptors, interleukin receptors, phagocyte receptors, immunoglobulin receptors, activation markers, transferrin receptors, members of the scavenger receptor cysteine-rich (SRCR) superfamily, growth factor receptors, selectins, MHC molecules, TNF-receptors, and TNF-R associated factors. Examples of typical B-cell surface proteins include β-glycan, B cell antigen receptor (BAC), B7-2, B-cell receptor (BCR), C3d receptor, CD1, CD18, CD19, CD20, CD21, CD22, CD23, CD35, CD40, CD5, CD6, CD69, CD69, CD71, CD79a/CD79b dimer, CD95, endoglin, Fas antigen, human Ig receptors, Fc receptor proteins (e.g., subtypes of Fcα, Fcγ, Fcε, etc.), IgM, gp200-MR6, Growth Hormone Receptor (GH-R), ICAM-1, ILT2, CD85, MHC class I and II molecules, transforming growth factor receptor (TGF-R), α4β7 integrin, αiibβ3 integrin, αLβ2 integrin, and αvβ3 integrin.

Where fibrocytes are the desired targets, the ligand can recognize a protein found on fibrocyte cell surfaces such as major histocompatability complex molecules, co-stimulatory molecules, and adhesion molecules, such as, for example, CD11a, CD13, CD34, CD45, CD54, CD58, CD80, CD86, HLA –DQ, HLA –DR, HLA-DP, and MHC-I and -II molecules. Of course, other cell types can be similarly targeted using domains known to be present within the surfaces of the desired cell.

In addition to the virion having the ligand, the complex also includes a (i.e., at least one) nucleic acid encoding a first non-native antigen. The antigen is "non-native" in that it is not present on a wild-type virus corresponding to the source from which the virion is derived. With this stricture in mind, the antigen can be

any moiety to which it is desired to effect an immune response. Typically, the antigen is an antigenic peptide derived from a pathogen (e.g., a virus, a bacterium, a spore or fungus, a parasite, etc.) or from a cancerous cell. Examples of some suitable antigens include, but are not limited to, viral antigens (e.g., from HIV GP120, Env, gag, pol or nef proteins or polypeptides, EBV latent membrane protein, etc.), endoglin, and tumor-associated antigens (TAAs) (e.g., BAGE, carcinoembryonic antigen (CEA), CASP-8, β-catenin, CDK-1, ESO-1, gp75, gp100, MAGE-1, -2, and -3, MART-1, mucins (MUC), MUM-1, p53, PAP, PSA, PSMA, ras, tyrosinase, trp-1 and –2, etc.). In another embodiment, an antigen can be a synthetic polypeptide (e.g., an HLA-A2-restricted gp100 peptide (see, e.g., Rosenberg *et al.*, *Nat. Med.*, *4*, 321-27 (1998)). For example, such an antigenic polypeptide can include a series of antigenic epitopes, such as between about 1 and about 15 (e.g., between about 5 and 10) such antigenic epitopes. Indeed, the antigenic properties of the inventive vaccine can be enhanced against that pathogen by using such synthetic antigens having epitope domains derived from the same pathogen. To facilitate processing and proper presentation, preferably each of such domains contains between about 5 and about 10 amino acids (e.g., about 8 amino acids) and/or is separated from other domains by polyamino acid spacers (e.g., polyalanine). Similarly, antigenicity also can be enhanced where the complex includes a plurality of such nucleic acids (i.e., encoding more than one first antigen).

Expression of the nucleic acid encoding the first antigen within the immune effector cells can permit the stimulation of an MHC-I response against the antigen. Moreover, in some embodiments, it is desirable for the complex to have a plurality of nucleic acids encoding antigens (e.g., to stimulate immune responses against multiple sites on a pathogen or to stimulate immune responses against multiple pathogens). Thus, the nucleic acid preferably is expressed within immune effector cells, and more preferably APCs. To facilitate expression, the nucleic acid encoding the first antigen is operably linked to a promoter that is active within the desired immune effector cell.

Preferably, in addition to the ligand and the nucleic acid encoding the first antigen, the complex also includes at least one second non-native antigen. This antigen can, as is the case for the ligand, be displayed on the surface of the virion, but it need not be so displayed (e.g., it can be a latent antigen), so long as it elicits an immune response. The second antigen can be any suitable antigenic moiety, such as those set forth above. The presence of the second antigen can facilitate an MHC-II response against the antigen. In many embodiments, to enhance the

potency of the immune response, the first (i.e., encoded) antigen(s) and the second (i.e., surface) antigen(s) are derived from the same pathogen. Such a strategy facilitates the generation of both an MHC-I and MHC-II response to the pathogen. In this respect, a particularly preferred embodiment is for at least one first antigen to be the same as at least one second antigen.

To facilitate attachment of the ligand to the desired cell, the ligand should be displayed on the surface of the virion within the complex. Suitable display can be accomplished by any desired means. For example, the ligand can be a domain present within a bi- or multi-specific protein (e.g., a bi-specific antibody), another domain of which selectively binds to a moiety present on the virion. In another embodiment, the ligand can be integrally connected to the virion. For example, the virion can include at least one chimeric protein having at least one first domain derived from a viral capsid protein and at least one-second domain comprising the ligand. Within such chimeric proteins, the viral capsid protein domains, as well as the ligand domains, can be full length or truncated. Of course, the virion can include a plurality of such proteins. In yet another embodiment, the ligand can be chemically conjugated to the virion by methods known in the art.

The virion is composed of viral capsid proteins, and it can be, but it need not be, an intact virus. Indeed, the virion can be a virus-like particle (VLP), empty capsid, or other viral-derived structure that has a lumen and a surface. However, preferably the virion is an intact virus, in which instance the nucleic acid encoding the first nucleic acid comprises a viral genome, typically also including encoding one or more of the viral capsid proteins making up the virion. Where the nucleic acid is a viral genome, it need not be complete, and, indeed, for many applications, the genome preferably contains one or more mutations interfering with viral replication. In some embodiments, the vector most preferably is replication incompetent except in packaging cells. Any suitable manipulation of the viral genome can be applied to achieve this end, many of which are known in the art. Thus, for example, where the virion and nucleic acid constitute an adenovirus, it can have replication-inactivating mutations, e.g., within the E1a, E1b, E2, and/or E4 regions of the genome. Similarly, where the virus is HSV, it can have inactivating mutations in one or more immediate early genes (e.g., ICP4, ICP27, ICP0) to render it replication incompetent. In other embodiments, for example where mimicry of a live virus is desired, it is desirable for the virus to be replication competent.

Structurally, the virion can be enveloped or non-enveloped, and it can be derived from any desirable type of virus. Preferably, the virion is derived from a

virus useful as a gene-transfer vector, such as, for example, herpesviruses (e.g., Herpes Simplex, Epstein-Barr, pox, etc.), adenoviruses, adeno-associated viruses (AAV), and other viruses known in the art. As considerable advances have been made in engineering functional chimeric adenoviral coat proteins, in embodiments in which ligands and/or second antigens are desired to be integral with the virion, preferably the virion is derived from an adenoviral capsid. In this respect, any chimeric protein desirably has at least one domain representing the ligand (or second antigen) and at least one other domain derived from an adenoviral coat protein (e.g., fiber, hexon, penton, pIIIa, pVI, pIX).

To further direct the virion to the desired immune effector cells, preferably its natural tropism for other types of cells is attenuated or eliminated. Methods suitable for attenuating native viral tropism are known in the art. For example, adenoviral-based virions can have one or more mutant adenoviral fiber protein(s) exhibiting reduced affinity for a native adenoviral cellular receptor (see, e.g., International Patent Application WO 98/54346 (Wickham *et al.*)). Moreover, an adenovirus-derived virion can include one or more recombinant penton base protein(s) lacking a native RGD sequence to reduce cell binding via α_v integrins (see, e.g., U.S. Patents 5,559,099 (Wickham *et al.*) and 5,731,190 (Wickham *et al.*)). Similarly, an adenovirus-derived virion can include one or more recombinant hexon(s) lacking a native sequence (e.g., HVR) to reduce its ability to be recognized by a neutralizing antibody (see, e.g., International Patent Application WO 98/40509 (Crystal *et al.*)). In other embodiments, a herpesvirus-derived virion can have mutant envelope glycoproteins lacking cell-surface ligands (see, e.g., International Patent Application WO 99/06583 (Glorioso *et al.*)).

Preferably, the immune response elicited against the complex is specific to either the first (i.e., surface) and/or second (i.e., encoded) antigen(s). Thus, the virion preferably elicits less virion-specific immunogenicity in a host animal than does a corresponding wild-type virion. To facilitate this, the virion can be engineered to lack a native immunodominant epitopes, such as are present in adenovirus, for example, on the viral hexon protein (see, e.g., International Patent Application WO 98/04509). Similarly, the virus can be engineered to lack one or more glycosylation or phosphorylation site(s). Alternatively, the virion can be conjugated to a lipid derivative of polyethylene glycol comprising a primary amine group, an epoxy group, or a diacylglycerol group. Without being bound by any particular theory, such modifications are believed to mask the virion, at least in part, from scavenging, particularly by the cells of the reticulo-endothelial system.

Regardless of whether the virion is an intact virus or a VLP, the complex is useful for delivering the nucleic acid to an immune effector cell such that the nucleic acid is expressed within the immune effector cell to produce the first antigen. To facilitate this, the complex can include adjunct constituents for facilitating transduction of the cells (e.g., one or more liposomes), and the presence of such adjuncts is preferred when the virion is other than an intact virus. To further promote immune activation, the complex can include at least one second nucleic acid sequence encoding a factor that activates an immune effector cell. For example, the factor can be a cytokine, such as granulocyte colony stimulating factor (CSF), monocyte CSF, granulocyte and monocyte CSF, interleukins 1-12, tumor necrosis factor-α or -β, macrophage inflammatory protein (MIP)-1-α or -β, MIP-2, interferon-γ (IFN-γ), etc. In embodiments where dendritic cells, macrophages, or other APCs are the targeted immune-effector cells, the factor can be CD40-L or osteopontin (or active fragments of these proteins), as these accentuate IL-12 and attenuate IL-10, which is associated with activation of the CD40 protein and the specificity of the response through initiation of the TH-1 and TH-2 subsets. Similarly, the second nucleic acid can encode a superantigen (e.g., a protein, such as are found on some bacteria, that prime cells of the immune system). In other embodiments, the second nucleic acid can encode an anticancer agent, e.g., a cytokine, an enzyme that converts a prodrug to a toxin (e.g., HSV tk), an apoptotic or an anti-angiogenic agent, many of which are known in the art.

The second nucleic acid can be present within a larger nucleic acid molecule that also includes the first nucleic acid (which is typically the case, where the capsid or virion includes an intact virus), or the two nucleic acid molecules can be within different nucleic acids, if desired. Of course, the second nucleic acid should be operably linked to a promoter sufficient to drive expression of the encoded factor within the desired immune effector cells, as described above. As with the first nucleic acid, any suitable promoter can be employed with respect to the second nucleic acid, such as a constitutive promoter (e.g., a viral immediate early promoter), a tissue-specific promoter, a regulatable promoter (e.g., metallothionin promoter, tetracycline-responsive promoter, RU486-responsive promoter, etc.), or other desired promoter. Indeed, the first and second nucleic acids can share the same promoter, such as, for example, separated by internal ribosome entry sites (IRES). In another embodiment, the first and second nucleic acids are on opposite sides of a strong bi-directional promoter, many of which are known in the art (see, e.g., Lee *et al.*, *Mol Cells.*, *10*(1), 47-53 (2000), Dong *et al.*,

J. Cell. Biochem., *77*(1), 50-64 (2000), and Li *et al.*, *J. Cell. Biochem.*, *273*(43):28170-7 (1998)).

The complex can be made by standard methods. Typically, the virions are constructed by producing viral capsid or coat proteins within packaging cells appropriate for the type of virus (e.g., HEK 293 cells, Vero cells, etc.) within which they will associate to form the virions. Ligands and antigens can be attached to, or incorporated into, the virions by known methods (see, e.g., U.S. Patents 5,965,541, 5,962,311, 5,846,782, 5,770,442, 5,731,190, 5,712.136, and 5,559,099). Of course, where the nucleic acid(s) are included within a viral genome, the genome will be packaged within the virion during syntheses. Conversely, where at least one nucleic acid is extragenomic, it is included within the complex by admixing it with the virion under suitable conditions, along with other components of the complex, such as liposomes (see, e.g., U.S. Patent 5,928,944).

The virions within the complex can be homogeneous, in which the antigens, ligands, capsids and nucleic acids of each member are identical or substantially identical. In other applications, the complex can be heterogeneous (i.e., a "library"). Indeed, the invention provides a library including a plurality of complexes, each comprising a virion as described above and at least a first nucleic acid encoding a first non-native antigen. The library is heterogeneous in that antigens of at least two of the plurality of the complexes are different. Of course, the virions within the library also can include ligands and other nucleic acids as described above (e.g., encoding antigens, cytokines, or other factors of interest). Typically, the library is constructed by generating a random or semirandom population of putative antigens that are variously associated with the virion as described above. For example, the putative antigens can be generated by proteolysis of proteins derived from a desired pathogen, the products of which can be fused to the capsid or to bi-specific molecules (as appropriate). Alternatively, mRNA or genomic DNA libraries from the desired pathogen can be randomly amplified, or digested, to result in fragments that can be subcloned into vectors suitable for inclusion within the complex. For association with the virion, for example, the fragments can be cloned in-frame with viral capsid proteins (including truncated derivatives thereof) to generate chimeric capsid proteins containing antigenic domains. Similarly, the fragments can be cloned so as to be operably linked to the promoter suitable for expression within the desired immune effector cells, as described above.

The inventive complex (or library) can be used to infect or transduce cells, typically immune effector cells. For delivery into a host animal, the complex (or library) can be incorporated into a suitable carrier to form a pharmaceutical or pharmacological composition. As such, the complex can be formulated and/or administered with a pharmacologically acceptable carrier (e.g., a pharmaceutically-acceptable carrier). Any suitable carrier is within the scope of the invention. The exact formulation, of course, depends on the nature of the desired application (e.g., cell type, mode of administration, etc.), and many suitable formulations are known in the art (see, e.g., U.S. Patent 5,559,099). Of course, the pharmaceutical composition can include other constituents, such as, for example, other agents that activate or enhance an immune response (e.g., (complete) Freund's reagent).

The complex, library, and pharmaceutical composition of the present invention are useful for eliciting immune responses within a desired immune effector cell, typically an APC. Thus, the invention provides a method of eliciting such a response using a complex comprising a virion and a first nucleic acid encoding a first non-native antigen. The immune response can be or comprise presentation of at least one of the antigens as an MHC-I-constrained moiety (an "MHC-I response") or as an MHC-II-constrained moiety (an "MHC-II response"). Preferably, the method results in both MHC-I and MHC-II responses. To promote both types of responses, preferably the virion has at least one second non-native antigen displayed on its surface, in addition to including the nucleic acid encoding the first antigen.

The method can be employed *in vivo*, typically within a mammal, or *ex vivo* or *in vitro* on isolated immune effector cells. For *in vitro* application, suitable cells (preferably APCs) can be isolated from the skin, spleen, bone marrow, other lymphoid organs, lymph nodes, or blood using methods known in the art (see, e.g., Unanue, *Fundamental Immunology*, Third Edition, Paul (ed.), Raven Press, Ltd.: New York., pp. 119-21 (1993); U.S. Patent 5,962,318). In other protocols, peripheral blood monocytes can be isolated and cultured to mature them into dendritic cells (see, e.g., Rossi *et al.*, *Immunol. Lett.*, *31*(2), 189-97 (1992)). After isolation and purification, the cells can be exposed to the inventive complex (or library) and maintained under conditions sufficient for them to develop an immune response (typically, incubation for about a day or two under appropriate culture conditions). To enhance antigen presentation, preferably the cells also are exposed to one or more activation factors (such as the cytokines discussed above). Such factors can be delivered exogenously, for example in the medium in which the

cells are cultured. Alternatively, where the complex includes a nucleic acid encoding such factors, its expression within the cells can effectively deliver the factor to the cells. In any event, following a suitable incubation, the cells can be monitored to assess their immune response by any suitable method (e.g., flow cytometry, FACS, elispot analysis, etc.). Thereafter, the cells can be propagated or maintained as desired. Such cells can be reintroduced into mammals *in vivo* for presenting the MHC-I and/or MHC-II antigens to immunize the mammal against the antigen(s) presented to the immune effector cells.

Where a library (such as described above) is employed, the method affords the ability to assess the antigenicity of at least one "test," or putative. antigen. The method is conducted similarly as described above, except that the relative strengths of the response to the test antigen(s) are compared within the population of cells. Typically, after exposure to the library, the cells are subjected to subcloning (e.g., by limiting dilution), plated separately, and then expanded before assessing the strength of the response to the library. Colonies exhibiting a strong response to the antigen can be analyzed to determine the identity of the antigen, which then can be employed as a vaccine.

When the method is applied *in vivo*, the invention provides a method of inoculating or immunizing an animal. In accordance with the method, a complex (or library), such as is set forth above, is introduced into the mammal under conditions sufficient for the mammal to mount an immune response to the antigens. While many methods of inoculation are known in the art, subdermal or subcutaneous injection is favored for many applications. For maximally targeting immune effector cells, especially *in vivo*, preferably the complex includes at least one ligand displayed on the surface of the virion, as described above, recognizing an epitope present on a desired type of immune effector cell, preferably an APC. The presence of the ligand permits the complex to be targeted preferentially to the immune effector cells. Also, preferably the virion is further engineered (as discussed above) to reduce any tropism that corresponding wild-type viruses might exhibit towards non-immune effector cells, to maximally direct the complex to the desired immune effector cells. To heighten the response of the immune effector cells within the mammal, preferably the complex further includes at least one nucleic acid sequence encoding a factor that activates the cell, such as those cytokines, CD40-L, or osteopontin, as described above. Expression of the nucleic acid, thus, activates the cell to heighten the immune response.

EXAMPLE

While one of skill in the art is fully able to practice the instant invention upon reading the foregoing detailed description, the following example will help elucidate some of its features. In particular, it describes the construction of an adenoviral vector having a non-native ligand recognizing an epitope present on an immune effector cell, a first nucleic acid encoding a non-native antigen, and a second nucleic acid encoding an activator of APCs. Of course, as this example is presented for purely illustrative purposes, it should not be used to construe the scope of the invention in a limited manner, but rather it should be seen as expanding upon the foregoing description of the invention as a whole.

The procedures employed in this example, such as gene cloning, vector manipulation, and cell culture are familiar to those of ordinary skill in this art (see, e.g., Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2d edition, Cold Spring Harbor Press (1989)). As such, and in the interest of brevity, experimental protocols are not recited in detail.

To construct the vector, a sequence encoding an RGD motif is engineered into the fiber gene of a replication-deficient adenovirus so as to fuse it to the HI loop of the encoded fiber, as previously described (see U.S. Patent 5,965,541). The RGD motif targets the virus to dendritic cells by binding cell-surface heparin molecules. Additionally, a hemagglutinin (HA) tag is engineered into the chimeric fiber adjacent to the RGD motif, which facilitates purification of the virus. This construct can be engineered within a plasmid encoding the fiber gene, the sequence of which is known.

The nucleic acids are introduced into the E1 region of the adenoviral genome as a recombinant expression cassette cloned into a plasmid encoding the E1 region of the adenoviral genome. This cassette includes a bi-directional promoter that directs transcription of two coding polynucleotides in opposite directions. In one direction, the promoter is operably linked to the gene encoding osteopontin (Ono *et al.*, *Mol. Immunol.*, *32*(6), 447-48 (1995)). In the other direction, the promoter is operably linked to a nucleic acid encoding a polyepitope "string of beads" antigen (see, e.g., Toes *et al.*, *Proc. Nat. Acad. Sci. (USA)*, *94*(26), 14660-65 (1997)).

Each of the desired constructs within plasmids are then recombined back into the "master-vector" adenoviral backbone using standard *E. coli* transformation and manipulation techniques. The master vector then is transfected into packaging cells by standard protocols and incubated for about 5-7 days. Because of the vector's altered tropism (by virtue of the manipulations to the fiber), it is desirable

to employ a cell expressing a ligand that can bind the HA tag in the fibers. One such suitable cell line, derived from HEK-293 cells (i.e., 293-HA cells), is known in the art (see, e.g., International Patent Application WO 98/54346). Following this initial incubation, the supernatant is collected and used to infect fresh packaging cells, which are again incubated for about 5-7 days. After several rounds of amplification, standard assays (e.g., Southern blots, hybridization, cell-binding, etc.) are used to analyze the resultant recombinant adenoviral vector.

A stock of the resultant vector then is exposed to cultured dendritic cells under conditions to facilitate infection of the cells. Following several days, the cells are processed to determine that the vectors have, in fact, infected them. The cells then are assayed, e.g., by Northern hybridization and/or Western blot, to determine that the nucleic acids encoding the osteopontin and the string of beads have been expressed. Another population of the cells is assayed to demonstrate that the string of beads is presented as an MHC-I-constrained epitope and that the HA epitope is expressed in a MHC-II-constrained manner. Yet another population of the cells is assayed for the effect of the osteopontin, e.g., by assaying for activation of the CD40 protein and/or for IL-12/IL-10 levels.

INCORPORATION BY REFERENCE

All sources (e.g., inventor's certificates, patent applications, patents, printed publications, repository accessions or records, utility models, world-wide web pages, and the like) referred to or cited anywhere in this document or in any drawing, Sequence Listing, or Statement filed concurrently herewith are hereby incorporated into and made part of this specification by such reference thereto.

INTERPRETATION GUIDELINES

The foregoing detailed description sets forth "preferred embodiments" of this invention, including the best mode known to the inventors for carrying it out. Of course, upon reading the foregoing description, variations of those preferred embodiments will become obvious to those of ordinary skill in the art. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law.

As used herein, singular indicators (e.g., "a" or "one") include the plural, unless otherwise indicated. The term "consisting essentially of" indicates that

unlisted ingredients or steps that do not materially affect the basic and novel properties of the invention can be employed in addition to the specifically recited ingredients or steps. In contrast, the terms "comprising" or "having" indicate that any ingredients or steps can be present in addition to those recited. The term "consisting of" indicates that only the recited ingredients or steps are present, but does not foreclose the possibility that equivalents of the ingredients or steps can substitute for those specifically recited.

WHAT IS CLAIMED IS:

1. A complex comprising (a) a virion having a surface and a lumen and comprising viral capsid proteins, (b) at least one non-native ligand displayed on the surface, which at least one ligand recognizes an epitope present on an immune effector cell, and (c) at least one first nucleic acid encoding at least one first non-native antigen.

2. The complex of claim 1, wherein at least one ligand recognizes a protein on an antigen presenting cell.

3. The complex of claim 1 or 2, wherein at least one ligand recognizes CD-40.

4. The complex of any of claims 1-3, wherein at least one ligand comprises an RGD motif or three or more tandem lysine and/or histidine residues.

5. The complex of any of claims 1-4, wherein an antigen is a gene product from a pathogen or a malignant cell.

6. The complex of any of claims 1-5, wherein an antigen is a synthetic polypeptide having from about 1 to about 15 antigenic domains.

7. The complex of any of claims 1-6, further comprising at least one non-native second antigen.

8. The complex of claim 7, wherein at least one first antigen is the same as at least one second antigen.

9. The complex of claim 7 or 8, wherein the virion comprises at least one chimeric protein comprising at least one first domain derived from a viral capsid protein and at least one second domain comprising at least one second antigen or at least one ligand.

10. The complex of any of claims 1-9, further comprising a liposome.

11. The complex of any of claims 1-10, wherein the virion is non-enveloped.

12. The complex of any of claims 1-11, wherein the virion elicits less virion-specific immunogenicity in a host animal than does a corresponding wild-type virion.

13. The complex of any of claims 1-12, wherein the virion comprises an adenoviral capsid.

14. The complex of any of claims 1-13, wherein the first nucleic acid comprises a viral genome.

15. The complex of any of claims 1-15, wherein the nucleic acid is expressed in an immune effector cell.

16. The complex of any of claims 1-15, further comprising at least one second nucleic acid sequence encoding at least one polypeptide that activates an immune effector cell.

17. The complex of claim 16, wherein at least one polypeptide comprises a domain derived from CD40-L or osteopontin.

18. The complex of claim 16 or 17, wherein at least one polypeptide is a cytokine.

19. A method of inoculating a mammal, the method comprising introducing the complex of claim 1 into a mammal under conditions sufficient for the mammal to mount an immune response to at least one first non-native antigen.

20. The method of claim 19, wherein the complex further comprises at least one second non-native antigen and where the animal mounts at least one immune response against at least one second non-native antigen.

21. A method of inoculating a mammal, the method comprising introducing a complex comprising (a) a virion having a surface and a lumen and comprising viral capsid proteins, (b) at least one first nucleic acid encoding at least one first non-native antigen, and (c) at least one second non-native antigen into a mammal under conditions sufficient for the mammal to mount at least one immune response to at least one of the antigens.

22. The method of claim 21, wherein the complex further comprises at least one non-native ligand displayed on the surface, which recognizes an epitope present on an immune effector cell.

23. The method of any of claims 19-22, wherein the mammal comprises an immune effector cell, and wherein at least one immune response comprises an MCH-1 response within the immune effector cell.

24. The method of any of claims 19-23, wherein the mammal comprises an immune effector cell, and wherein at least one immune response comprises an MCH-2 response within the immune effector cell.

25. The method of any of claims 19-24, wherein the complex comprises at least one second nucleic acid sequence encoding at least one polypeptide that activates an immune effector cell, which is expressed within the mammal under conditions sufficient to activate the immune effector cell.

26. The method of any of claims 19-25, wherein at least one polypeptide comprises a domain derived from CD40-L or osteopontin.

27. The method of any of claims 19-26, wherein at least one polypeptide is a cytokine.

28. A library comprising a plurality including at least two of complexes of claim 1, wherein at least one of the first non-native antigens of at least two of the plurality of the complexes are different.

29. A library comprising a plurality including at least two complexes, each of which comprises (a) a virion having a surface and a lumen and comprising viral capsid proteins and (b) at least one first nucleic acid encoding at least one first non-native antigen, wherein at least one of the first non-native antigens of at least two of the plurality of the complexes are different.

30. The library of claim 29, wherein at least one of the complexes further comprises at least one non-native ligand displayed on the surface, which recognizes an epitope present on an immune effector cell.

31. The library of claim 29 or 30, wherein at least one of the complexes further comprises at least one second non-native antigen.

32. A method of assessing the antigenicity of at least one test antigen, the method comprising (a) exposing the library of any of claims 29-31 to a population of immune effector cells, the library containing least one test antigen, (b) maintaining the cells under conditions such that the cells develop a response to the test antigen, and (c) assessing the relative strength of the response of the cells to the test antigen.

33. A pharmaceutical composition comprising (a) a complex which comprises (i) a virion having a surface and a lumen and comprising viral capsid proteins, (ii) at least one first nucleic acid encoding at least one first non-native antigen, and (b) a physiologically-acceptable carrier.

34. The pharmaceutical composition of claim 33, wherein the complex further comprises at least one second non-native antigen

35. The pharmaceutical composition of claim 33 or 34, wherein the complex further comprises at least one non-native ligand displayed on the surface, which recognizes an epitope present on an immune effector cell.

36. The pharmaceutical composition of any of claims 33-35, wherein the complex comprises at least one second nucleic acid sequence encoding a polypeptide that activates an immune effector cell.

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 01/03892

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/00 C12N7/01 C12N15/861 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, EMBASE, MEDLINE, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 15638 A (LUDWIG INST CANCER RES) 16 April 1998 (1998-04-16) page 6, line 18 -page 7, line 27 page 8, line 4 -page 8, line 26 page 10, line 24 -page 10, line 25 page 13, line 31 -page 14, line 18 page 16, line 24 -page 16, line 32 page 17, line 24 -page 17, line 32 page 19, line 10 -page 19, line 20 page 37, line 43	1,2,4-9, 11-16, 18-25, 27,33-36
Y	claims 16-20;25	26

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

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'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 May 2001	22/06/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Renggli, J

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 01/03892

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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